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EXPRESSION OF HUMAN INTERLEUKIN 2, HUMAN TUMOR NECROSIS FACTOR ALPHA AND MOUSE INTERFERON GAMMA IN *MYCOBACTERIUM BOVIS* BCG

BY

DEQUAN KONG

©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY

Edmonton, Alberta

Fall, 1995

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Date July 28, 1995

"God's eternal power and character cannot be seen. But from the beginning of creation, God has shown what these are like by all he has made. That's why those people don't have any excuse."

---- HOLY BIBLE: Roman 1, 20

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled EXPRESSION OF HUMAN INTERLEUKIN 2, HUMAN TUMOR NECROSIS FACTOR ALPHA AND MOUSE INTERFERON GAMMA IN *MYCOBACTERIUM BOVIS* BCG submitted by DEQUAN KONG in partial fulfillment of the requirements for the degree of DOCTOR of PHILOSOPHY.

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DEDICATED TO

MY FATHER KONG LINBEI

MY MOTHER XIAO YUNZHEN

MY WIFE YANG GUANGZHEN

MY DAUGHTER LISA WEIJIA KONG

FOR THEIR LOVE AND ENCOURAGEMENT

ABSTRACT

Mycobacterium bovis BCG, an attenuated strain of *M. bovis*, is the most widely used vaccine for the prevention of tuberculosis (TB) in the world. A number of features of BCG make it an attractive vaccine vehicle. In this thesis, I report the expression of human interleukin-2 (huIL-2), human tumor necrosis factor alpha (huTNF-α) and mouse interferon gamma (mIFN-γ) in BCG in order to deliver functional cytokines to enhance the host immune response.

Two recombinant BCG (rBCG) lines, BCG-pMV306-CI (huIL-2) and BCG-pMV306-CII (huIL-2) secreting biologically active huIL-2, were constructed from the Pasteur strain of BCG. In both rBCG lines, the expression plasmids were integrated into the chromosome and therefore, stably maintained. These rBCG lines showed limited growth in BALB/c mice compared to the control BCG. Our preliminary studies suggest that this inhibition of growth *in vivo* is likely due to the secreted huIL-2.

Biologically active huTNF-α was expressed intracellularly to a relatively high level (about 2.5 μg/10 mg rBCG cells) in one of the rBCG line BCG-pMV261-CIII (huTNF-α). The effects of initiation codon change on the gene expression in BCG was studied using huTNF-α as a reporter gene. I found that the expression of huTNF-α, fused to BCG hsp60 regulatory sequences, was increased at least 100 percent when the AUG initiation codon of the hsp60 gene was changed to GUG. This increased expression is accompanied by an increase in huTNF-α specific mRNA. This is the first example

showing that GUG is used as a more efficient initiation codon than AUG.

mIFN-γ was expressed in two rBCG lines, BCG-pMV261-CI (mIFN-γ) and BCG-pMV306-CIII (mIFN-γ). While the mIFN-γ, detected by ELISA from the cell sonicates of both rBCG lines, was confirmed by a bioassay, no biological activity of mIFN-γ could be detected from the supernatants of both rBCG cultures. It was found that the mIFN-γ is not stable in the Dubos-ADC medium under the BCG growth conditions. When testing the effects of our mIFN-γ-secreting BCG recombinant on the *L. major* infection in BALB/c mice, we found that vaccination with BCG-pMV261-CI (mIFN-γ) could either protect or exacerbate the *L. major* infection, depending on the time interval between the vaccination and infection.

In addition, as a preliminary project to learn cytokine bioassays and recombinant expression techniques, I perfomed *in vitro* mutagenesis on two N-glycosylation sites of mouse interleukin 5 (mIL-5) to make it more homogenous for analysis of mIL-5 spatial structure by X-ray diffraction. The mutated mIL-5, expressed in a baculovirus system, showed more homogeneity than the wild type mIL-5, but retained the same biological activity. From one of the mutated mIL-5, two crystal forms were obtained, diffracting to beyond 2.5 A resolution. The study of the mIL-5 spatial structure is in progress.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
Amp^r	Ampicillin resistance
APC	Antigen Presenting Cell
aph	Aminoglycoside phosphotransferase
BCG	Bacillus Calmette-Guérin
BMM	Marrow-derived Macrophages
bp	base pair
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CMI	Cell-mediated Immunity
CTL cell	Cytolytic cell
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FDA	Fluorescein diacetate
HCl	Hydrochloric acid
HIV-1	Human Immunodeficient Virus 1
hsp60	heat shock protein 60
huTNF-α	Human Tumor Necrosis Factor alpha
IL-2	Interleukin 2

IL-5	Interleukin 5
k b	kilobase
KCl	Potassium Chloride
L. major	*Leishmania major*
LB	Luria-Bertani medium
LMP	Low melting Point
LPS	Lipopolysaccharide
M. leprae	*Mycobacterium leprae*
M. tuberculosis	*Mycobacterium tuberculosis*
MAC	*Mycobacterium. avium* Complex
MCS	Multiple Cloning Sites
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
MHC	Major Histocompatibility Complex
mIFN-γ	Mouse Interferon gamma
mmol	millimole
MP	Mononuclear Phagocytes
NaCl	Sodium Chloride
NaH_2PO_4	Sodium phosphate monobasic
NaOH	Sodium Hydroxide
OspA	Outer Surface Lipoprotein A
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PhoS	Phosphate-binding Protein
rBCG	recombinant BCG
RNA	Ribonucleic Acid
RNI	Reactive Nitrogen Intermediate

ROI	Reactive Oxygen Intermediate
SDS	Sodium Dodecyl Sulphate
sf9 cell	*Spodoptera frugiperda* 9 cell
SSC	Sodium Chloride, Sodium Citrate
TE	Tris-EDTA buffer
Tris	THAM-Tris (Hydroxyethyl) amino methane
WHO	World Health Organization
µg	microgram
µl	microliter

Chapter I

General Introduction

My major project deals with the expression of the cytokines human interleukin 2 (huIL-2), human tumor necrosis factor alpha (huTNF-α) and mouse interferon gamma (mIFN-γ) in *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG). In this chapter, I will review the biology and molecular genetics of mycobacteria, immunity to mycobacterial infection and various aspects of the three cytokines (IL-2, TNF-α and IFN-γ).

1. Mycobacteria

A. General characteristics of mycobacteria

The mycobacteria, comprising the genus *Mycobacterium*, are aerobic, nonmotile, and rod-shaped bacteria with two distinguishing characteristics: acid-fastness and slow growth (reviewed in Grange, 1988; Wayne and Kubica, 1994).

The mycobacterial cell has a very thick and complex cell wall made up of complex lipids and mycolic acid, which is responsible for the acid-fast staining property (Draper, 1982). This cell wall is not readily stainable by common staining procedures (Gram's Staining), so that mycobacteria can not be classified as Gram positive or Gram negative bacteria. Mycobacterial cell walls can be stained by some special procedures, such as heating in a saturated solution of a basic dye fuchsin. Once stained the mycobacterial cell wall is resistant to decolorization by 3% hydrochloric acid in ethanol, which can decolorize nearly all bacterial cells (Bishop and Neumann, 1970).

Mycobacteria grow very slowly compared to other bacteria (Grange, 1988). The generation time for a mycobacterium under optimal conditions is generally 14-15 hours; therefore, 2-3 weeks are

required to produce visible colonies. The genus *Mycobacterium* has been divided into two major groups according to the relative growth rate: the slow and rapid growers. Slow growers, such as *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG, require more than 7 days to produce easily seen isolated colonies under optimal culture conditions. For rapid growers, colonies can be seen in less than 7 days under comparable conditions. For example, *Mycobacterium smegmatis* needs only 2 days to produce visible colonies.

The genus *Mycobacterium* has been divided into more than 42 species (Grange, 1988), most of which are environmental saprophytes, with the exception of *M. tuberculosis* and *M. leprae*. A detailed description of the different species of the genus *mycobacterium* is available in Bergey's manual (Wayne and Kubica, 1994).

B. Pathogenic mycobacteria and their associated diseases

Mycobacteria have long been recognized to be important pathogens worldwide. *M. tuberculosis*, the causative agent of tuberculosis, infects more than 10 million people each year and causes 3 million deaths annually, especially in the developing countries (Styblo, 1989). Leprosy, caused by the infection of *M. leprae*, affects 10.5 million people and while rarely fatal, it causes deformity in its sufferers (Grange, 1988). Species such as *M. marinum*, *M. ulcerans*, *M. avium*, *M. intracelluare* and *M. xenopi* are opportunistic pathogens, which can cause serious infections in people

with the acquired immunodeficiency syndrome (AIDS) and other immunocompromised individuals (Quinn, 1989; Horsburgh, 1991).

Tuberculosis in humans is mainly caused by the tubercle bacillus *M. tuberculosis*, though it is occasionally caused by other members of the *M. tuberculosis* complex including *M. bovis*, *M. microti* and *M. africanum* (Grange, 1988). While the lung is the most frequently affected organ (pulmonary tuberculosis), the disease may involve virtually any organ or system of the body (non-pulmonary tuberculosis). The bacteria enter the lung through the inhalation of airborne droplets containing *M. tuberculosis*. If a person has not previously been infected with *M. tuberculosis*, he/she may develop primary tuberculosis. Among generally healthy people with a normal cell-mediated immune response, primary tuberculosis is usually mild and most of time (about 90%) asymptomatic (reviewed in Hopewell, 1994). In this case, only a positive tuberculin skin test indicates the infection with *M. tuberculosis* (Sbarbaro, 1986). Conversely, in some immunocompromised people who do not develop sufficient cell-mediated immunity, the bacteria may invade the blood stream and spread to almost any organ, resulting in disseminated miliary tuberculosis. Years after contracting primary tuberculosis, some people develop secondary tuberculosis with characteristic tuberculosis symptoms such as chronic fever, weight loss, night sweats and productive cough often with hemoptysis. Secondary tuberculosis is mainly due to the reactivation of the dormant tubercle bacilli in the body, although the disease may be caused by reinfection with external bacteria. Reactivation of *M. tuberculosis* is probably due to the transient impairment of the cell-mediated

immune response, and it is often associated with malnutrition, alcoholism, diabetes, old age or infection with other bacteria or some viruses, such as measles and HIV (Balasubramaniau, 1994).

Leprosy (or Hansen's disease), caused by infection with *Mycobacterium leprae*, primarily affects the skin, mucous membranes and nerves (reviewed in Jopling, 1984). *M. leprae* was first identified in 1874 by the Norwegian physician G. A. Hansen and it is one of the first bacteria found to be associated with a human disease. *M. leprae* still cannot be grown in artificial medium or tissue culture *in vitro*. Currently, it is grown in the footpads of mice or in the nine-banded armadillo (Lumpkin *et al.*, 1983). One of the remarkable features of leprosy is that it presents as a spectrum of clinical manifestations that correlates with immune responses to the pathogen (Ridley and Jopling, 1966). There are two polar clinical manifestations of the disease, lepromatous leprosy and tuberculoid leprosy, with intermediate forms also occurring (reviewed in Midlin, 1993). In patients with tuberculoid leprosy, the number of lesions is few although tissue and nerve damage is frequent; while in patients with lepromatous leprosy, numerous lesions occur throughout the skin. The lesions of tuberculoid leprosy are characterized by very few bacilli but many lymphocytes and granulomas containing mature epithelioid cells. In contrast, lesions of lepromatous leprosy contain few if any lymphocytes but numerous bacilli within immature macrophages. It has been suggested that the different clinical manifestations of leprosy are associated with the development of cell-mediated immune response to *M. leprae*. In patients with tuberculoid leprosy, an active cell-mediated immune (CMI) response

to *M. leprae* develops while the CMI response in lepromatous leprosy is suppressed (reviewed in Bloom, 1986).

C. *Mycobacterium bovis* BCG

Mycobacterium bovis BCG, attenuated from a *Mycobacterium bovis* strain by *in vitro* passage between 1906 and 1919 (Calmette *et al.*, 1924), is one of oldest and most widely used vaccines. Initially used as a vaccine against tuberculosis, BCG has been found to act as a general immunostimulant (Groves, 1993), and it has been used in the treatment of some cancers and has some potential use in the treatment of certain infectious diseases (Grimald *et al.*, 1980; Fortier *et al.*, 1987). BCG has recently been approved by the USA Food and Drug Administration for the treatment of superficial bladder cancers.

The first trial of BCG vaccination against tuberculosis in humans was carried out by Weill-Halle in 1925 (Weill-Halle, 1925). BCG vaccination was encouraged worldwide during the 1950s by the World Health Organization (WHO), and has now become the most widely used vaccine in the world. However, the systematic evaluation of BCG vaccines by 10 randomized controlled trials revealed that the protective efficacy against tuberculosis varied in different populations (ranging from 0 to 80%) (reviewed in Fine, 1989). Different mechanisms have been proposed to explain these divergent results, such as different BCG strains used in different trials, different *M. tuberculosis* substrains with different virulence in different regions, and genetic differences in trial populations (Lugosi, 1992). Despite extensive research, the reason for the inconsistency

of the efficacy of BCG vaccination remains unknown and control of tuberculosis requires a new vaccine.

Based on Pear's observation that cancer and tuberculosis have an inverse relationship, the first trial using BCG in the treatment of cancer was carried out by Holmgran in 1935 (Holmgran, 1935). BCG has now become the treatment of choice for recurrence of superficial bladder tumors.

The exact mechanisms of BCG's antitumor activity remains unclear, but it seems that close contact between the tumor cells and BCG is necessary (Stover and Peter, 1980; Herr and Whitmore, 1987). The attachment of BCG to tumor cells is mainly mediated by the interaction of antigen 85A of BCG and the fibronectins on the surface of tumor cells (Ratliff *et al.*, 1987; Kavoussi *et al.*, 1990). Two main theories have been proposed to explain the mechanisms of action of BCG on tumor cells: one is the direct cytotoxicity of BCG cells or certain BCG cellular components to the tumor cells (Rajala *et al.*, 1992), the other is that BCG acts on tumor cells through the stimulation of the host immunological response (Bohle *et al.*, 1990; El-demiry *et al.*, 1987). Although direct cytotoxicity of BCG to some tumor cells has been demonstrated *in vitro* (Rajala *et al.*, 1992), it is generally believed that BCG acts on tumor cells mainly through the induction of a nonspecific immune reaction, which kills tumor cells as "innocent bystanders". For example, it has been found that BCG has no effect against tumor growth in nude mice, but that antitumor activity can be restored by adoptive transfer of BCG sensitized T cells (Ratliff *et al.*, 1987).

Cytokines probably play an important role in the BCG antitumor effects. IFN-γ, IL-1, IL-2 and TNF-α have all been reported to be present in the urine of the patients treated with BCG (Presco *et al.*, 1990; Bohle *et al.*, 1990). After intravesical BCG treatment, T lymphocytes, macrophages and polymorphonuclear leukocytes are the main cells infiltrating the tumor tissue of the urinary bladder (De Boer *et al.*, 1991; Leong *et al.*, 1990). This indicates that these cells probably play a role in the BCG antitumor activity.

D. Immunity to mycobacterial infection

Acquired immunity against mycobacterial infection is primarily cell-mediated even though specific antibodies are produced during mycobacterial infections (reviewed in Chan and Kaufmann, 1994; Barnes *et al.*, 1994). This conclusion is based on the observations that immunity can only be transferred by sensitized lymphocytes, not by serum (Orme and Collins, 1983). Many experiments indicate that the major cells involved in antimycobacterial immunity are mononuclear phagocytes (MP) and T lymphocytes. The activated MP serve as the main effector cells while the T cells act as the principle inducers of protection, but at the same time, both MP and T lymphocytes play a major role in the pathogenesis of mycobacterial infection. MP provides the habitat for the mycobacteria and survival of this pathogen while the T cells and their secreted cytokines also contribute to the destruction of tissues. Therefore, the clinical outcome of infections with mycobacteria depend on the coordinated cross-talk between MP and T cells.

1). The role of mononuclear phagocytes (MP)

Pathogenic mycobacteria (*M. tuberculosis*, *M. bovis* and *M. leprae*) are facultative intracellular bacteria and MP are the main habitat. Even though *M. tuberculosis* can infect a variety of mammalian cells *in vitro* (Sheppard, 1958), it can be found only in polymorphonuclear leukocytes and MP in the infected tissues (Filley and Rook, 1991).

The preferential localization of tubercle bacilli inside macrophages indicates that macrophages must play a critical role in the host defense against *M. tuberculosis* infection. Macrophages probably perform three important functions in the host defense against mycobacterial and other intracellular bacterial infections (Kaufmann, 1993). First of all, as major antigen presenting cells, macrophages can process and present mycobacterial antigens to T cells, which can induce the proliferation of specific CD_4 T lymphocytes (Germain, 1993). The CD_4 T cells and their secreted cytokines are the central players in the acquired resistance to *M. tuberculosis* infection. Second, macrophages can produce a set of cytokines, including IL-1, IL-6, IL-10 and tumor necrosis factor alpha (TNF-α) in response to *M. tuberculosis* infection (Valone *et al.*, 1988; Toossi *et al.*, 1991; Barnes *et al.*, 1992). All of these cytokines play an important role in both immunoprotection and immunopathogenicity in mycobacterial infections (Ding *et al.*, 1988; Bermudez and Young, 1988). The third critical role that macrophages play is that after induction and activation by some cytokines, macrophages can produce many proteolytic enzymes and

metabolites, such as reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), that exhibit mycobactericidal effects (for review, see Liew and Cox, 1991; Nathan, 1992). Killing of the intracellular pathogens by activated macrophages is the main effector function in the cell-mediated immunity against intracellular infections.

2). The role of T lymphocytes

T lymphocytes play a central role in the acquired resistance to a mycobacterial infection. AIDS patients suffering from T cell deficiencies have a high incidence of tuberculosis (Barnes *et al.*, 1991). Nu/nu and scid mice, deficient in T cells, suffered more severely from experimental *M. tuberculosis* and BCG infections than normal mice (Sher *et al.*, 1975; Izzo and North, 1992).

Based on the type of receptor present on the T cell surface, T lymphocytes have been divided into two broad populations, α/β T cells with α and β polypeptide chains as their receptor, and γ/δ T cells with γ and δ chain as their receptor (Davis and Bjorkman, 1988). α/β T cells make up more than 95% of all T cells in the secondary lymphoid organs and peripheral blood of humans and experimental animals while γ/δ T cells constitute a minor T cell population (Raulet, 1989). Furthermore, α/β T cells are subdivided into two distinct subgroups (CD_4 T cells and CD_8 T cells) based on their cellular surface markers and their functions. CD_4 T cells recognize antigenic peptides in the context of major histocompatibility complex (MHC) class II molecules and normally perform a helper function for B cells, cytolytic cells (CTL) and other lymphocytes. CD_8 T cells recognize

peptides in the context of MHC class I molecules and generally function as CTL cells (for review, see Germain, 1993).

CD_4 T cells play a dominant but not exclusive role in the immune response against mycobacterial infection. Mice depleted of CD_4 T cells by specific monoclonal antibodies are incapable of controlling the growth of BCG and suffer a more serious infection with *M. tuberculosis* (Muller *et al.*, 1987; Pedrazzini *et al.*, 1987). In addition, protection against tuberculosis can be adoptively transferred using CD_4 cells isolated from sensitized animals (Orme and Collins, 1984). MHC class II gene knock-out mice lacking functionally active CD_4 T cells die of BCG and sublethal *M. tuberculosis* infections (Mombaerts *et al.*, 1992; 1993). In humans, depletion of CD_4 T cells in HIV infection markedly increases susceptibility to *M. tuberculosis* and other opportunistic mycobacterial infections (Barnes *et al.*, 1991).

CD_8 T cells probably play a substantial role in the protection against tuberculosis. Depletion of CD_8 T cells with specific monoclonal antibodies worsen *M. tuberculosis* infection in mice, and selected CD_8 T cells transferred protection against tuberculosis (Orme and Collins, 1984; Muller *et al.*, 1987). Consistent with this finding, knock-out mice lacking functional CD_8 T cells because of the disrupted $\beta 2$-microglobulin gene suffer more serious *M. tuberculosis* infection (Flynn *et al.*, 1992). In addition, mycobacterial-specific CD_8 T cells have been isolated from *M. tuberculosis* and BCG infected mice and the isolated CD_8 T cells can directly lyse *M. tuberculosis*-infected cells *in vitro* (Delibero *et al.*, 1988)

Several lines of indirect evidence suggest that γ/δ T cells probably play a role in the initial immune response to *M. tuberculosis* infection. In mice, γ/δ T cells accumulate early at the site of BCG infection and the number of γ/δ T cells is markedly increased in draining lymph nodes and lungs after primary infection with *M. tuberculosis* (Janis *et al.*, 1989; Augustin *et al.*, 1989) Moreover, severe combined immunodeficient (SCID) mice suffer more serious BCG infection than nu/nu mice and mice depleted of CD_4 and CD_8 T cells which provides evidence for a role of γ/δ T cells (Izzo and Nuth, 1992) However, direct proof of the roles of γ/δ T cells in mycobacterial infection is still lacking.

3). The role of cytokines

Evidence described in the last two sections indicates that T cells and macrophages are two major players in the immune defense against a mycobacterial infection. Both T cells and macrophages, especially CD_4 T helper cells, function largely through their secreted cytokines. The roles that cytokines play in the host immunity to mycobacterial or other intracellular bacterial infections are illustrated by both *in vitro* and *in vivo* experimental approaches (for review, see Kaufmann, 1993). The *in vivo* approaches include: 1) administration of cytokine-specific neutralizing antibodies; 2) direct injection of various recombinant cytokines before or after mycobacterial infection; 3) infection of knock-out mice lacking defined cytokines with various mycobacterial species. The combined use of these experimental approaches indicates that cytokines play a

central role in the resistance to mycobacterial infection. In this review, I will concentrate on the roles that three cytokines (IL-2, IFN-γ and TNF-α) play during mycobacterial infection.

a. Interferon gamma (IFN-γ)

As a potent macrophage activator and immunoregulator, IFN-γ plays a central part in the host resistance to mycobacterial and other intracellular bacterial infections (for review see, Andrew and Lowrie, 1988). It has been shown by Rook *et al.* (1986) that murine peritoneal macrophages and bone marrow-derived macrophages (BMM) can restrict the growth of *M. bovis* and *M. tuberculosis in vitro* after activation with rIFN-γ. Administration of rIFN-γ protects mice from a lethal *M. tuberculosis* infection while neutralization with anti-IFN-γ antibodies markedly exacerbates the infections (Denis, 1991). IFN-γ knock-out mice are unable to control a normally sublethal dose of *M. tuberculosis* infection (Cooper *et al.*, 1993; Flynn *et al.*, 1993). While many experiments indicate that IFN-γ plays an essential role in the protective immunity to *M. tuberculosis* infection in mice, the role IFN-γ plays in human mycobacterial infection is less well understood. First, *in vitro* stimulation of human MP with IFN-γ to kill or inhibit the growth of *M. tuberculosis* and *M. bovis* produced conflicting results from different research groups (Rook *et al.*, 1986; Douvas *et al.*, 1985). Second, the production of RNI, the major effector of bacteriostatic/bacteriocidal MP activities, has not been shown unequivocally in human MPs (Denis, 1991; Sherman *et al.*, 1991; Padgett *et al.*, 1992). Recent experiments indicated that 1,25-dihydroxyvitamin D3 is needed as a costimulator for human MP to

maximally inhibit the growth of mycobacteria (Crowle *et al.*, 1987; Rook, 1988).

b. Tumor necrosis factor alpha (TNF-α)

As a major mediator of inflammation and a coactivator of macrophages, TNF-α plays an important role in mycobacterial infections (reviewed in Flesch and Kaufmann, 1993). Human mononuclear phagocytes secrete large amount of TNF-α after stimulation with *M. tuberculosis*, mycobacterial protein or lipoarabinomannan isolated from *M. tuberculosis* cell wall (Wallis *et al.*, 1990; Ogawa *et al.*, 1991; Moreno *et al.*, 1989). High TNF-α levels are also detected in pleural fluids of patients with tuberculous pleuritis (Barnes *et al.*, 1990). Although TNF-α alone fails to induce antimycobacterial activity on murine macrophages *in vitro*, it shows marked synergy with IFN-γ (Flesch and Kaufmann, 1990). Continuous infusion of TNF-α increases the resistance of BALB/C mice to a lethal dose of *M. tuberculosis* H37Rv infection and infusion of antibodies against TNF-α markedly enhances susceptibility of naive mice to tuberculosis (Denis, 1991). Neutralization of TNF-α *in vivo* has been shown to greatly enhance mouse susceptibility to *M. bovis* BCG infection, and conversely, direct infusion of TNF-α *in vivo* can increase resistance against a virulent *M. avium* strain (Bermudez *et al.*, 1989). Furthermore, TNF-α has been shown to play a crucial role in granuloma formation (Kindler *et al.*, 1989). Administration of anti-TNF-α antibodies inhibits granuloma formation, and consequently exacerbates the mycobacterial infection. On the other hand, high TNF-α production during chronic tuberculosis may be

responsible for the development of cachexia, also referred to as tuberculous consumption (Kaufmann, 1993).

c. Interleukin-2 (IL-2)

IL-2 plays a role in the host resistance against mycobacterial infection although the role of IL-2 is less well studied compared to IFN-γ and TNF-α. TNF-α alone or in combination with IL-2 can activate human and murine macrophages to kill *M avium* complex (MAC) *in vitro* (Bermudez and Young, 1988). Treatment of MAC infected mice with TNF-α and/or IL-2 significantly reduces the number of viable bacteria in the blood, liver and spleen of the infected animals (Bermudez *et al.*, 1989). In addition, administration of recombinant IL-2 can limit the replication of *M. lepraemurium* and *M bovis* BCG in mice (Jeevan and Asherson, 1988).

E. Molecular genetics of mycobacteria

Studies on the genetics of mycobacteria lags far behind those in other bacteria. Many reasons have been given to explain why the genetic studies on mycobacteria, which contain two very important human pathogens, have been largely neglected. One reason is the lack of appropriate genetic tools. While the "gene knockout" technique, involving the replacement of a wild-type gene with a nonfunctional mutated gene by homologous recombination, has been widely used to study gene functions in both eukaryotic and prokaryotic cells, this powerful technique has not been used in mycobacteria due to the very low frequency of homologous recombination in slow growing mycobacteria (Coston and Davis, 1994). Homologous recombination, which is used as a genetic tool for

creating specific mutations and mapping genes in other bacteria, has only been reported in the fast-growing mycobacteria, *M. smegmatis* (Husson *et al.*, 1990) Three widely used techniques (transformation, transduction and conjugation) for gene transfer in bacteria have only been studied quite recently in mycobacteria (for review, see McFadden, 1990). Moreover, mycobacteria are the most difficult bacteria to manipulate experimentally due to their slow-growth rate and their tendency to clump (discussed in IA). In addition, the biological hazard associated with the manipulation of pathogenic *M. tuberculosis* and *M. leprae* has deterred many researchers. On the other hand, the development of powerful recombinant DNA technology opens up a new era for the studies of molecular genetics of different mycobacterial species. Since the first report of cloning and characterization of mycobacterial genes encoding the protein antigens of *M. leprae* (Clark-Curtiss *et al.*, 1985; Young *et al.*, 1985), more than 50 genes have been cloned from various mycobacterial species (Young and Cole, 1993). The genome project will sequence the whole genome of *M. tuberculosis* and *M. leprae*. Many genetic techniques for introducing and expressing foreign genes in mycobacteria have been developed during recent years with the aim of developing BCG as a vaccine vehicle (Aldovini and Young, 1990). In this section, I will review some of the progress, which includes cloning of genes from various mycobacterial species, the mycobacterial genome project, the regulation of gene expression in mycobacteria and various genetic techniques available for expression of foreign genes in mycobacteria.

1). Genome structure of mycobacteria

Studies on the genome structure of mycobacteria began with the determination of the Guanosine and Cytosine (G+C) content of mycobacterial DNA and estimation of the genome sizes (Clark-Curtiss, 1990). The G+C content of mycobacterial DNA is generally higher compared to other bacteria, ranging from 64 to 70% (Wayne and Gross, 1968; Bradley, 1973; Clark-Curtiss *et al.*, 1985) with the exception being *M. leprae* having a G+C content of 58% (Imaeda *et al.*, 1982). Most mycobacteria have a large genome relative to other prokaryotes, in the range of 3.1 to 4.4 x 10^9 Da (Bradley, 1973; McFadden *et al.*, 1987). It is interesting that the pathogenic mycobacteria have smaller genomes than the other mycobacterial species: the genome of *M. leprae*, *M. tuberculosis* and *M. bovis* is 2.2 x 10^9, 2.5 x 10^9 and 2.8 x 10^9 Da respectively (Clark-Curtiss *et al.*, 1985; Bradley, 1973).

During the last 10 years, the application of recombinant DNA technology allowed more than 50 genes from various mycobacterial species to be cloned and in some cases, the function of the genes to be determined (Young and Cole, 1993). The most widely applied techniques for cloning mycobacterial genes is the use of the lambda gt11 system (Young *et al.*, 1985; Andersen *et al.*, 1988). In this system, λgt11 expression libraries constructed from various mycobacterial species are screened using labeled mycobacterial protein-specific monoclonal antibodies or some polyclonal antisera from mycobacteria-immune animals or from patients infected by mycobacteria.

When the N-terminal sequences of the purified protein is available, another approach is generally used. In this approach, synthetic oligonucleotide probes are used to screen a recombinant DNA library. The genes cloned by this approach are mainly those encoding secreted proteins which are present in the culture medium and therefore, easily purified (Harboe *et al.*, 1986; Matsuo *et al.*, 1988). rRNA genes from various mycobacterial species were cloned by using an *E. coli* rRNA gene as a probe (Bercovier *et al.*, 1986). This approach takes advantage of the fact that rRNA genes are generally conserved throughout the whole prokaryotic kingdom. The cloning of the rRNA genes from different species of mycobacteria reveals that there is only one copy of the rRNA gene in the genome of the slow growing mycobacteria, while two copies of rRNA genes are present in the fast growing mycobacterial species (Bercovier *et al.*, 1989). This observation has been used as an explanation for the slower growth rate of the slow-growing mycobacteria (Sela *et al.*, 1989). Cloning of the citrate synthase gene from *M. leprae* was based on the complementation of a citrate synthase mutation of an *E. coli* strain with a clone from a cosmid pYA626: *M. leprae* library (Jacobs *et al.*, 1986). Even though many genes have been cloned by the approaches described above, the function of most cloned genes is still unknown. Only in some situations, the function of the cloned genes is identified by sequence analysis and comparison with other bacteria. Notably, a number of immune dominant antigens have been identified as the members of the conserved heat shock protein families, such as the heat shock 60 and 70 protein genes (Mehra *et al.*, 1992; Young *et al.*, 1988; Shinnick, 1987).

The mycobacterial genome project aims to sequence the whole genome of *M. leprae* and *M. tuberculosis* (Cole and Smith, 1994). Several cosmids from *M. leprae* and *M. tuberculosis* have already been completely sequenced (Honore *et al.*, 1993). The first step in these genome projects is to construct contig maps of *M. leprae* and *M. tuberculosis* chromosome (Eiglmeier *et al.*, 1993; Cole and Smith, 1994). Contig maps have been made from the analysis of cosmid libraries carrying large DNA fragments from the chromosome of *M. tuberculosis* H37Rv and *M. leprae*. At the same time, the physical maps of these two pathogens will be made using rarely cutting restriction enzymes and pulsed-field gel electrophoresis (PFGE) (Cole and Smith, 1994). Eventually, the sequence of a set of selected cosmids with minimal overlaps will be determined using some large-scale sequencing technologies (Cole and Smith, 1994). Sequencing of the whole genome of *M. tuberculosis* and *M. leprae* will provide a wealth of information, which will be useful for identifying the virulent genes involved in the pathogenesis of these two important pathogens and for designing new rational drugs and vaccines for the control and final eradication of leprosy and tuberculosis.

2). Gene expression and regulation in mycobacteria

The regulation of gene expression in bacteria is a complex process and the mechanisms involved may vary with different genes and with different bacteria (Lewin, 1994). However, the basic principles involved in the regulation of gene expression seem to be similar among *E. coli* and other well-studied bacteria (Doi, 1991). The transcriptional and translational machinery are quite similar

among different bacteria and the signals (the promoter sequences, ribosomal binding sites and various other trans- and cis- regulatory elements) involved in the transcription and translation are often conserved (Reznikoff *et al.*, 1985; Gottesman, 1984). The various regulatory mechanisms involved in gene expression in bacteria are best studied in two different bacterial species, Gram positive *Bacillus subtilis* and Gram negative *E. coli*. Even though the mechanisms involved in the regulation of gene expression in mycobacteria are largely unknown, some evidence indicates that the basic mechanisms are also similar to those in other bacteria (for review see Dale and Patki, 1990). First of all, most cloned mycobacterial genes seem to have a typical prokaryotic promoter and ribosomal binding site (Dale and Patki, 1990). Second, some mycobacterial genes, such as the genes encoding the biotin carrier proteins of various mycobacterial species (Collins *et al.*, 1987), and the gene for the *M. tuberculosis* 38 Kd antigen (Andersen *et al.*, 1988), can be expressed in *E. coli* presumably using the mycobacterial transcription and translation signals. The BCG heat shock 60 gene was cloned by screening a BCG genomic library, constructed in an *E. coli* pBR322 vector, using a monoclonal antibody (Shinnick, 1987; Thole *et al.*, 1987; Mehra *et al.*, 1986). Moreover, a citrate synthase mutation of *E. coli* can be complemented by a clone from an expression library of BCG (Jacobs *et al.*, 1986).

a. Control of transcription initiation

In *E. coli* and other well-studied bacteria, it appears that a single type of RNA polymerase is responsible for the synthesis of all three types of RNAs (mRNA, rRNA and tRNA) (Doi, 1991). The RNA

polymerase from *E. coli* is the best characterized RNA polymerase. It contains four types of subunits ($\alpha 2\beta\beta'\sigma$) and has a molecular weight of about 480,000 daltons (Burgess *et al.*, 1987). The complete enzyme or holoenzyme ($\alpha 2\beta\beta'\sigma$) can be separated into two functional components: the core enzyme ($\alpha 2\beta\beta'$) and the sigma (σ) factor. The core enzyme is capable of synthesizing RNA on a DNA template, but can not initiate transcription at the proper sites. The sigma factor ensures that the bacterial RNA polymerase binds stably to DNA only at the proper sites (promoter). After the transcription extends 8-9 bases, sigma factor is released. The specific recognition of promoters by RNA polymerase, mainly mediated by sigma factor, plays a critical role in the regulation of the transcriptional start site, efficiency and the direction. In *E. coli* and many other bacteria, transcription of most genes during normal vegetative growth requires a predominant or primary σ factor. In *E. coli* the primary σ factor is referred to as σ_{70} and in *B. subtilis* σ_{43}. The primary σ factor recognizes similar consensus sequences of the promoters centered at -35 (TTGACA) and at -10 (TATAAT), which are usually separated by 17-19 bases. In many bacterial species studied, there exists some alternative σ factors that control the transcription of coordinately regulated sets of genes under certain conditions (Doi and Wang, 1986). The consensus sequences in the promoter of these genes are quite distinct from those recognized by the primary σ factor. For example, in *E. coli* σ_{32} is activated in response to heat shock (Taylor *et al.*, 1984; Grossman *et al.*, 1984) and σ_{54} is expressed in response to nitrogen starvation (Hirschman *et al.*, 1985; Hunt and Magasanik, 1985). It is only recently that several studies on the mycobacterial RNA polymerase

and sigma factors were reported. Sequencing of the *M. leprae* chromosome DNA (*M. leprae* genome project) has identified two genes that show homology to rpoB and rpoC of *E. coli* and probably encode the β and β' subunits of the *M. leprae* RNA polymerase (Honore *et al.*, 1993). RNA polymerase from the fast-growing mycobacterial strain *M. smegmatis* has recently been purified and shown to function *in vitro* to transcribe a mycobacterial gene (Levin and Hatfull, 1993). By using a DNA probe derived from the gene encoding the primary sigma factor of *Streptomyces coelicolor*, Predich *et al.* (1995) identified and cloned two genes that probably encode the sigma factors of *M. smegmatis*.

While the studies on the RNA polymerase and the sigma factors and their roles in the regulation of gene expression in mycobacteria are only in their preliminary stages, the studies are concentrated on identifying the putative consensus sequences involved in the transcription and translation of mycobacterial genes. The putative promoter sites of most cloned mycobacterial gene were identified by searching for homology to *E. coli* promoter sequences. The results are reviewed in (Dale and Patki, 1990). Are the putative promoters really functional as promoters in mycobacteria? The transcriptional start sites have been mapped by primer extension only in two cases; one for the hsp60 gene and another for the hsp70 gene of BCG (Stover *et al.*, 1991; Burlein *et al.*, 1994). No other studies have been reported. The roles that these putative promoters play in mycobacterial gene expression need further study by some well-established techniques, such as S1 nuclease mapping, primer extension and, ultimately, by direct RNA polymerase binding studies.

In addition to the regulatory mechanisms described above, transcription of some genes in bacteria is under the control of various positive and /or negative regulatory elements (Lewin, 1994). A classic example is the regulation of gene expression in the lactose operon in *E. coli*. This kind of regulation mechanism may also exist in mycobacteria. For example, the DNA sequence of *M. tuberculosis* 38 kDa antigen shows a significant degree of homology to the *E. coli* phosphate-binding protein Pst S (also known as PhoS), which suggests the possibility that it is a phosphate-regulated gene (Andersen *et al.*, 1988; Andersen and Hansen, 1989). However, this type of regulation has not been directly demonstrated in mycobacteria.

b. Post-transcriptional regulation

Overall, post-transcriptional controls play a minor role in the regulation of bacterial gene expression relative to the control of transcription. However, there are some mRNA sequences that are required for efficient expression or may control the level of expression.

In *E. coli* and other well-studied bacteria, translation is normally initiated following the binding of ribosomes to a specific ribosomal binding site (the translation equivalent of promoters) on the mRNA. The ribosomal binding site (RBS) is characterized by the presence of a purine (A+G) rich sequence of about seven bases, called the Shine/Dalgarno sequence, followed after an interval of 4-7 bases, by a start codon (usually AUG). The binding of ribosomes to the RBS is thought to be facilitated by the complementary base pairing between the 3' end of the 16S ribosomal RNA and the Shine/Dalgarno

sequence on the mRNA. A computer search of the sequences of the cloned mycobacterial genes revealed the presence of putative ribosomal binding sites in most of them (reviewed in Dale and Patki, 1990), but whether the putative RBSs really function as ribosomal binding sequences is still unknown and their role in the regulation of gene expression in mycobacteria is also unclear.

One interesting feature concerning the translational initiation in mycobacteria is that GUG is used as a start codon nearly as frequently as AUG (Dale and Patki, 1990), which probably reflects the high G+C content of the mycobacterial DNA. The use of GUG is inferred based on the open reading frame of the cloned mycobacterial genes, and has only been confirmed by comparison with N-terminal amino acid analysis in a few cases (Yamaguchi *et al.*, 1988; Baird *et al.*, 1989).

3). Development of BCG into a vaccine vehicle

With the advent of recombinant DNA technology, many attenuated live viruses, such as vaccinia and adenovirus, and bacteria, such as BCG and salmonella, have been proposed to be used as vaccine vehicles to express and deliver foreign antigens to the immune system. Of all these proposed viral and bacterial vaccine vehicles, BCG has many advantages (Aldovini and Young, 1990). First of all, BCG is one of the oldest vaccines used in humans, and has been given to 2.5 billion people worldwide since 1948. It has proven to be very safe (Lotte *et al.*, 1984). Occasionally, vaccination with BCG is associated with some complications, but the availability of antibacterial drugs helps to avert serious effects. Second, BCG is one

of the few vaccines, which can be given at birth. A single dose of BCG vaccination can give long-lasting immune responses. Third, as a potent adjuvant and a general immune stimulant, BCG has been licensed as a treatment in bladder cancer. The adjuvant activity of BCG can enhance the immune response to recombinant proteins expressed in BCG. Finally, BCG can be given orally and is relatively inexpensive to manufacture, which is a very important factor for vaccines to be used worldwide especially in the developing countries.

Researchers have recently developed a variety of molecular genetic tools for expression of foreign genes in BCG. In this section, I am going to review some of these developments.

a. Vectors for expressing foreign gene in BCG

i. The mycobacteriophage-based vectors

The first generation of vectors used in mycobacteria were derived from the mycobacteriophages. The mycobacteriophages were first described in 1947 (Gardner and Weiser, 1947). Since then, over 250 mycobacteriophages have been found in various mycobacterial species, and mycobacteriophages have been used for many years to type and classify mycobacterial species (reviewed in Hatfull and Jacobs, 1994). Some phages have only recently been modified to act as vectors for the efficient transfer of genes to mycobacteria.

By insertion of an *E. coli* cosmid DNA fragment into the mycobacteriophage TM4, L1 and L5 DNA, several vectors, termed "shuttle phasmids" have been constructed (Jacobs *et al.*, 1987; Snapper *et al.*, 1988). They can replicate in *E. coli* as plasmids and

grow as phages in mycobacteria, which allows transfer of DNA between *E. coli* and mycobacteria.

Shuttle plasmid vectors based on the temperate phage, such as L1 and L5, allow the foreign genes cloned in the vector to be integrated, and therefore stably maintained in the mycobacterial chromosome (Snapper *et al.*, 1988). With the identification of sequences required for site-specific integration of the temperate phage into the chromosomal attB site, an integrative plasmid vector *E. coli*/BCG pMV361 was constructed (Stover *et al.*, 1991). The vector pMV306 used in my research was derived from pMV361, by replacing the expression cassette with a multiple cloning site (MCS).

ii. Plasmid-based vectors

Since the first description of a plasmid in mycobacteria in 1979 (Crawford and Bates, 1979), many studies have been performed on the plasmids of *M. avium* complex (*M. avium* and *M. intracellulare*), *M. scrofulaceum* and the *M. fortuitum* complex. No plasmids have been identified in the TB complex (For review see, Falkinham III and Crawford, 1994). The most extensively studied mycobacterial plasmid is pAL5000, which was carried by a fast-growing *M. fortuitum* strain and was originally described by Labidi *et al.* (1984). Based on this plasmid, several shuttle plasmid vectors capable of replicating in both *E. coli* and slow-growing mycobacteria such as BCG were constructed. Molecular genetic studies on the replication of pAL5000 localized the replicon to a 1.8-kb segment of pAL5000 DNA (Labidi *et al.*, 1992). The *E. coli*/BCG shuttle expression vector

pMV261 used in my studies was constructed using this defined 1.8 kb replicon (Stover *et al.*, 1991).

Even though some extrachromosomal plasmid vectors such as pMV261 have proven to be remarkably stable in the absence of any antibiotic selection (Stover *et al.*, 1991), most of the extrachromosomal plasmid vectors are not stable in the absence of selection. This is a potential problem when a recombinant BCG vaccine is used *in vivo* where antibiotic selection for plasmid maintenance is not possible. To obtain stable gene expression, it is preferred that gene expression cassettes are integrated into the BCG chromosome. Foreign genes could be integrated into the mycobacterial chromosome with the use of three different strategies, including homologous recombination (Husson *et al.*, 1990), site-specific recombination using the temperate mycobacteriophage L5-based vectors (Stover *et al.*, 1991) and DNA transposition elements (Dellagostin *et al.*, 1993). In some situations, integration may provide a way to maintain and express some genes which cannot be maintained using extrachromosomal plasmids because of over-expression lethality (See Chapter II, this thesis).

iii. Promoters used in expression vectors

The promoters of the heat shock proteins (hsp60 and hsp70) are most often used to drive expression of foreign genes in mycobacteria for the following reasons (Stover *et al.*, 1991; Aldovini and Young, 1991). First, these genes are among the first mycobacterial genes cloned, and their function is among the best studied. The transcriptional start sites for both hsp60 and hsp70

genes have been mapped by primer extension (Stover *et al.*, 1991). Second, although the expression of these genes is greatly enhanced in response to heat shock or other stress, their expression is constitutive under all growth conditions (Young *et al.*, 1990). Third, the expression of heat shock protein in other intracellular bacteria is induced upon infection of macrophages (Buchmeier and Heffron, 1990), which is the main habitat of mycobacterial replication. The use of these promoters can drive the expression of some foreign genes to 10% or more of total mycobacterial proteins (Stover *et al.*, 1991). Other promoters have also been used in directing the expression of foreign genes in BCG, including the promoters of genes encoding the *M. kansasii* alpha antigen (Matsuo *et al.*, 1990), the *Streptomyces albus* groES/groEL1 (Winter *et al.*, 1991), the *M. paratuberculosis* IS900 ORF2 (Murray *et al.*, 1992) and the *M. tuberculosis* 19-kDa antigen (Stover *et al.*, 1993).

b. Selectable markers

Since the introduction of DNA into mycobacteria is an inefficient process even with the use of electroporation, selectable genetic markers are required for the selection of mycobacterial cells that have taken up the DNA.

The most widely used marker for selection of transformation in mycobacteria is the aminoglycoside phosphotransferase (aph) gene from Tn5 or Tn903 of *E. coli*, which confers Kanamycin resistance (Snapper *et al.*, 1988). Expression of the aph gene seems to be under the control of its own expression signals. Therefore, the aph genes can be directly put into the expression vector without further

manipulation. In addition, Kanamycin is stable over the long incubation period required for the growth of the slow-growing mycobacteria. Other antibiotic resistance genes that have been used are those conferring resistance to Sulfonamide (Martin *et al.*, 1990), Hygromycin (Radford and Hodgson, 1991) and Chloramphenicol (Snapper *et al.*, 1988).

The eventual goal of expressing foreign genes in BCG is to use these recombinant BCG vaccines in humans. In this situation, use of antibiotic selectable markers may be a problem since the antibiotic resistance genes present in BCG could be transmitted to other pathogenic mycobacterial strains. One alternative strategy of selection is the use of the immunity functions of mycobacteriophage L5 gene71 (Donnelly-Wu *et al.*, 1993). The L5 gene71, cloned in a plasmid vector, can prevent mycobacterial cells from lysis by mycobacteriophages which are used to infect the transformed mycobacterial cells. Another useful strategy for nondrug selection is the development of vectors containing genes capable of complementing mycobacterial auxotrophic mutations. This has proven to be an effective strategy for selecting and maintaining plasmids in other bacteria but has not been achieved in slow-growing mycobacteria (Donnelly-Wu et al., 1993).

c. Introduction of foreign DNA into mycobacterial cells

The mycobacteria are naturally competent, but the frequency of natural transformation is very low. The initial method for introduction of foreign DNA into mycobacteria was the production and transformation of spheroplasts (Jacobs *et al.*, 1987). By this

method, the efficiency for transforming into fast-growing *M. smegmatis* is very low, possibly due to the difficulty of regenerating viable cells from spheroplasts. Transformation of slow-growing mycobacteria by this method has not yet been achieved. The highest efficiency for transformation of mycobacteria was obtained by electroporation (Jacobs *et al.*, 1991). In this method, the mycobacterial cells suspended in a high resistance electroporation buffer (10% glycerol) are subjected to high voltage electroporation (time constants reach 15.0 to 25.0 msec). Transformation efficiency by electroporation can reach 10^4-10^6 colonies/μg DNA (using a 10 kb plasmid).

d. Expression of foreign genes in BCG

Since the first report of expression of the aph gene from Tn903 in BCG (Snapper *et al.*, 1988), many foreign genes, ranging from the β-galactosidase gene of *E. coli* to the gag, pol and nef genes of HIV-1 have been expressed in BCG.

In 1990, Matsuo *et al.* reported the expression of a fusion protein involving an 8-amino acid epitope of HIV-1 p17 gag and the alpha antigen of BCG. Then several groups in 1991 reported the expression of gag, pol, env and nef genes of HIV-1 in BCG (Stover *et al.*, 1991; Aldovini and Young, 1991; Winter *et al.*, 1991; Murray *et al.*, 1992). All of these studies demonstrated that delivery of foreign antigens by rBCG to mice can elicit both humoral and cellular immune responses. Moreover, recent studies indicate that proteins delivered by rBCG could also protect some experimental animals from certain infectious diseases. Recombinant BCG expressing a

nontoxigenic fragment of tetanus toxin can elicit sufficient antibody production to protect mice from a lethal dose challenge of toxin (Cassatt *et al.*, 1993). Expression of a major surface protein gp63 of *L. major* by rBCG can elicit cell-mediated immune responses that can protect both CBA and BALB/C mice from cutaneous leishmaniasis (Connell *et al.*, 1993).

2. Cytokines (IL-2, IFN-γ and TNF-α)

Cytokine is a term given to a group of cell regulatory proteins produced by a wide variety of animal cells, which have originally been named lymphokines (mainly produced by T cells), monokines (mainly produced by mononuclear phagocytes), interleukins and interferons (For reviews see, Balkwill and Burke, 1989; Durum and Oppenheim, 1993; Howard *et al.*, 1993). Cytokines are proteins or glycoproteins, mostly in the molecular weight range of 5 Kd to 50 Kd. They exhibit their functions through binding to specific receptors on target cells and/or on the cytokine-producing cells themselves. Cytokines play an important role in many immunological and physiological responses, are involved in the pathophysiology of a range of diseases, and show great therapeutic potential. In this section, I will give a brief review on the proteins, genes and activities of three cytokines, IL-2, IFN-γ and TNF-α. The roles that these three cytokines play in the mycobacterial infection has been discussed in the first section.

A. Interleukin 2

Interleukin 2, originally designated T cell growth factor (TCGF), was first reported in 1976 by Morgan *et al.* They found that supernatant prepared from an *in vitro* cell culture of mitogen-stimulated human peripheral blood leukocytes contained an activity that could maintain normal human T cells without antigen for up to 9 months. Soon after this discovery, IL-2 was demonstrated to have pleiotropic effects on a number of cells and play a pivotal role in the host immune response to viral, bacterial and possibly tumor antigens (reviewed in Smith, 1988).

IL-2 is mainly produced by activated T lymphocytes (predominantly by CD_4 T cells and in less quantities by CD_8 T cells) (Smith, 1988). Natural human IL-2 is a 14-17 kDa glycoprotein, but the glycosylation is not needed for its biological activities (Robb, 1984; Wang *et al.*, 1984). Human IL-2 cDNA was cloned in 1983 (Taniguchi *et al.*, 1983) and the nucleotide sequence predicted a polypeptide of 153 amino acids including a 20 amino acid signal peptide, which is removed during the secretion of IL-2.

The availability of recombinant human and murine IL-2 makes it possible to carry out many *in vitro* and *in vivo* experiments. IL-2 is the major autocrine growth factor for T lymphocytes (de la Hera *et al.*, 1985; Lakhanpol *et al.*, 1987) and it also stimulates synthesis of other T cell-derived cytokines such as IFN-γ and lymphotoxin. IL-2 has been shown to enhance the *in vitro* induction of cytolytic T cells (Gillis *et al.*, 1979) and the *in vitro* induction of natural killer cell activity through induction of IFN-γ expression. The action of IL-2 on

T cells is mediated by binding to the IL-2 receptor (Minami *et al.*, 1993). In addition, IL-2 stimulates the growth of NK cells (Chun *et al.*, 1985) and enhances their cytolytic function (Savary *et al.*, 1989), producing so-called lymphokine-activated killer (LAK) cells. Induction and injection of the *in vitro* activated LAK cells is the basis of adoptive immunotherapy for some cancers (reviewed in Rosenberg and Lotze, 1986; Lotze and Rosenberg, 1988).

B. Interferon gamma (IFN-γ)

The interferons are a family of inducible proteins, that have the capability to protect cells from viral infection (reviewed in Langer and Pestka, 1985). The interferons are divided according to their biochemical characteristics into three classes: IFN-α, IFN-β and IFN-γ. The IFN-α and IFN-β (formerly known as type-I interferons) are produced by leukocytes and fibroblasts respectively in response to the viral infection of cells. Both IFN-α and IFN-β bind to the same receptor and share many characteristics. Both are acid (pH 2) and heat stable (56°C for 30 minutes). On the other hand, IFN-γ, also known as type II IFN or immune IFN, is primarily produced by some subsets of CD_4 T cells (such as Th1 T helper cells) and CD8 T cells in response to antigen or mitogen stimulation. IFN-γ is also produced by natural killer (NK) cells after IL-2 stimulation. IFN-γ is acid (pH 2) and heat labile (56°C for 30 minutes) (Mulkerrin and Wetzel, 1989).

The cloned human IFN-γ cDNA encodes a 166 amino acid polypeptide with the first 23 amino acids serving as signal peptide (Gray *et al.*, 1982). The predicted molecular weight of the

recombinant IFN-γ is 15,900 daltons. Human IFN-γ is a glycoprotein with two potential N-glycosylation sites. Under denaturing conditions, the fully glycosylated IFN-γ has a molecular weight of 25 kDa (Scahill *et al.*, 1983; Yphantis *et al.*, 1987). The natural human IFN-γ isolated from induced peripheral blood leukocytes is heterogeneous in size and charge with molecular weights ranging from 40 kDa to 60 kDa, and is thought to be a homodimer (Nagate *et al.*, 1987; Greelund *et al.*, 1992). The cDNAs for bovine, mouse and rat have been cloned by using human IFN-γ cDNA as a probe (Gray *et al.*, 1983). The mouse IFN-γ only shows 40% homology with the human IFN-γ, which provides an explanation for the species-specificity of IFN-γ.

Although IFN-γ was initially identified on the basis of its antiviral activities (Wheelock, 1965), IFN-γ has been found to be an extremely pleiotropic cytokine (Farrar and Schreiber, 1993). It is involved in regulating many immunologic and inflammatory processes and plays an important role in the host defense against nonviral infections especially against intracellular pathogens. IFN-γ is one of the major cytokines involved in the activation of macrophages and monocytes (Adams and Hamilton, 1984; Murray *et al.*, 1985; Pace *et al.*, 1983; Beaman *et al.*, 1992). IFN-γ activated macrophages show enhanced tumoricidal and microbicidal activities, which is mainly mediated by the increased production of ROIs and RNIs. IFN-γ can upregulate class II MHC antigens on several antigen presenting cells (APCs) (reviewed in Trinchieri and Perussia, 1985); consequently, this upregulation could augment and accelerate the

immune response. In addition, IFN-γ plays an important regulatory role by inducing the proliferation of Th1 T cells and inhibiting the proliferation and effector functions of Th2 T cells (Swain *et al.*, 1991; Gajewski *et al.*, 1989; Mosmann and Coffman, 1989). The consequence of this regulation is the down-regulation of humoral immunity and upregulation of cell-mediated immunity. This regulation plays a critical role in host defense against many intracellular pathogen infections, such as *leishimania major* (Sher and Coffman, 1992), and possibly *M. leprae*. and *M. tuberculosis* (Flesch and Kaufmann, 1993).

C. Tumor necrosis factor alpha

Tumor necrosis factor alpha was originally identified based on its ability to induce the necrosis of some mouse tumors both *in vivo* and *in vitro* (Old, 1985). It was independently discovered as "cachectin", a factor associated with the hypertriglyceridemia and wasting in rabbits chronically infected with *Trypanosoma brucei* (Beutler and Cerami, 1988). Tumor necrosis factor/cachectin was later named TNF-α while lymphotoxin was named TNF-β. Although they share only 30% homology, they have an identical action and bind to the same receptor (reviewed in Vassalli, 1992).

Although a variety of cells can produce TNF-α following appropriate stimulation, TNF-α is mainly produced by monocytes/macrophages after stimulation with LPS or various other exogenous or endogenous factors (Stein and Gordon, 1991; Chensue *et al.*, 1989). LPS is the most powerful TNF inducer, and can act on

monocytes and macrophages of all tested sources. Infection of macrophages with *M. tuberculosis* and *M. bovis* BCG leads to production of TNF-α (Rook *et al.*, 1987; Valone *et al.*, 1988).

Human TNF-α cDNA predicts a polypeptide of 233 amino acids with an unusually long (76 amino acids) signal peptide, which is removed during secretion of mature TNF-α (Pennica *et al.*, 1984). The natural human TNF-α is thought to function as a homotrimer with a molecular weight of 51 kDa (Smith and Baglioni, 1987; Jones *et al.*, 1989).

TNF-α is one of the most pleiotropic cytokines (for reviews see, Fiers, 1991; Vassalli, 1992). It binds to specific receptors present on virtually all types of nucleated cells and causes a vast array of changes in cell behavior. TNF-α plays an important role in host protection against bacterial and viral infection, but on the other hand, is also involved in the pathogenesis of many diseases. Different functions and effects of TNF-α depends on the cells and tissues stimulated.

Generally, TNF-α is not cytotoxic to most normal cells, but it can rapidly kill a large variety of human or mouse tumor cell lines. The mechanisms of tumor killing are currently not understood, but the cell death appears to resemble apoptosis with the characteristic DNA fragmentation of the target cells (Yamauchi *et al.*, 1989; Watanabe *et al.*, 1988). Killing is greatly enhanced by inhibiting protein synthesis by exposure of tumor cells to actinomycin D or cycloheximide (Kirstein and Baglioni, 1986). Therefore, it has been proposed that continuous synthesis of certain proteins protects normal cells from the TNF-α attack, whereas the susceptible tumor cells lack this

defense or repair mechanism (Wong *et al.*, 1988). One protein, manganese-dependent superoxide dismutase, has been implicated in this protective mechanism (Wong *et al.*, 1989).

TNF-α is one of the major inflammatory mediators (Vassalli, 1992). The inflammatory actions of TNF-α are dependent on its quantity. At low concentrations (approximately 10^{-9}M), TNF-α acts locally as a paracrine and autocrine regulator of leukocytes and endothelial cells to induce a local inflammatory reaction (Munro *et al.*, 1989). If the stimulus for TNF-α production is so strong that greater quantities of TNF-α are produced, TNF-α enters the blood stream and acts as an endocrine hormone, which results in a systemic inflammatory reaction. In this reaction, TNF-α mediates cachexia (wasting), which results in part from TNF-α's ability to suppress lipoprotein lipase synthesis and activity in adipocytes (Kawakami *et al.*, 1982). In addition, TNF-α is an endogenous pyrogen, which acts on cells in the hypothalamic regulatory region of the brain to induce fever (Dinarello *et al.*, 1986).

In addition, TNF-α can exhibit many other functions by acting on different cell types. One of the most important functions is the activation of macrophages (Liew *et al.*, 1990; Wirth and Kierszenbaum, 1988; Esparza *et al.*, 1987) and induction of macrophage differentiation (usually requiring or strongly synergized by IFN-γ).

3. Delivery of cytokines by BCG recombinants: Rationale for expression of cytokines in BCG

As described above, cytokines are a group of proteins or glycoproteins involved in communication between cells, mainly those of the immune system (Balkwill and Burke, 1989). Cytokines, induced as part of the host immune response to different types of bacterial, viral and parasitic infections, determine not only the magnitude but also the types of immune responses (Flesch and Kaufmann, 1993; Bogdan *et al.*, 1993). Notably, murine T helper cells have been divided into two subsets (Th1 and Th2) based on the cytokines produced (Mosmann and Coffman, 1989). Immunity induced by an infectious agent is determined, in part, by the pattern of cytokines secreted by different subsets of T helper cells (Mosmann and Moore, 1991; Locksley and Scott, 1991). Th1 cells, which produce IL-2, IFN-γ and lymphotoxin, stimulate the cell-mediated immune response; Th2 cells, which secrete IL-4, IL-5 and IL-6, generally augment the humoral immune response. Cytokines, produced by the Th1 cells, play a critical role in the host defense against intracellular infections like *M. tuberculosis* (Orme *et al.*, 1993), *M. leprae* (Modlin, 1993) and *L. major* (Locksley and Scott, 1991); while the humoral immune response augmented by the Th2 cell-secreted cytokines is effective against soluble toxins and some types of extracellular bacteria (Mosmann and Moore, 1991).

Considering the important roles cytokines play during the host immune response, it is not surprising that attempts have been made to modify the immune response by administration of recombinant

cytokines. For example, cytokines such as IL-2 and IFN-γ, have been used in clinical trials for the treatment of a variety of malignancies (Rosenberg *et al.*, 1985; Rosenberg *et al.*, 1988). IL-2, IFN-γ and TNF-α have been suggested for the treatment of various bacterial infections like tuberculosis (Bermudez and Kaplan, 1995), leprosy (Modlin, 1994) and leishmaniasis (Bogdan *et al.*, 1993). With the emergence of multiple-drug-resistant (MDR) strains of mycobacteria, it may be important to develop an immune therapy for the mycobacterial diseases caused by these MDR strains (Cole, 1994; Bottger, 1994). In addition, some cytokines such as IL-1, IL-2 and IFN-γ have been used as immunological adjuvants to improve the efficacy of some vaccines (for a review, see Heath and Playfair, 1992).

All of the above approaches using the direct administration of cytokines have the following drawbacks. Most cytokines have a very short half-life *in vivo*; high dose administration of most cytokines is generally associated with toxicity; it is difficult to target cytokines to the sites of immune reactivity (reviewed in Howard *et al.*, 1993). To overcome these problems, some strategies have been developed to deliver functional cytokines to the host, and in some way to prolong the *in vivo* half-life of the cytokines.

One of these approaches is the use of liposomes to gradually release the entrapped cytokines. Cytokines like IL-1, IL-2, IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF) and IFN-γ have been encapsulated in the multilameliar liposomes. The cytokines entrapped in the liposomes show longer half-lives *in vivo*

than the free cytokines (Anderson *et al.*, 1994). Mbawuike *et al.* (1990) showed that immunization of BALB/c mice with a mixture of IFN-γ entrapped in liposomes and an influenza A vaccine resulted in enhanced protection against influenza A infection compared to the immunization with the mixture of free IFN-γ and vaccine. *L. donovani*-infected BALB/c mice treated with mIFN-γ encapsulated in liposomes had significantly fewer liver parasites than the control mice treated with the same dose of free mIFN-γ (Hockertz *et al.*, 1991).

Another approach is the use of live vaccine (virus and bacteria) to express and deliver functional cytokines to the host immune system. Many cytokines have been expressed in vaccinia virus (VV) (for a review see, Ramshaw *et al.*, 1992). One of the most important findings from these studies is that production of IL-2, TNF-α or IFN-γ by vaccinia virus led to the control of VV replication and resolution of infection in nude mice or sublethally irradiated mice (Karupiah *et al.*, 1990; Sambhi *et al.*, 1991; Ramshaw *et al.*, 1987). Coexpression of IL-2 with a glycoprotein D (gD) of HSV-1 in Herpes simplex virus type-1 (HSV-1) virus increased the host immune response against gD protein (Hinuma *et al.*, 1991). Expression of the Th2 type cytokine, IL-4, in an attenuated aroA *Salmonella typhimurium* strain promotes the growth and survival of the bacteria in BALB/c mice (Denichet *et al.*, 1993).

With the advent of recombinant technology for expression of foreign genes in mycobacteria, BCG has been proposed as a live multivaccine vehicle. BCG has a long history of safe use and an ability to act as an adjuvant. Delivery of cytokines by recombinant

BCG to a host provides a novel means to enhance the host immune response. The secreted cytokines may act as immunological adjuvants to stimulate both humoral and cell-mediated immune responses against mycobacterial or other recombinant proteins coexpressed in BCG to improve vaccine efficacy. O'Donnell *et al.* (1994) reported the expression of mouse IL-2 in recombinant BCG. They found that the IL-2 expressing-BCG can enhance the mouse interferon gamma (mIFN-γ) production by splenocytes *in vitro*. One of the advantages of using BCG instead of liposomes to deliver cytokines to the host immune system is that BCG can persist longer *in vivo* and therefore, gradually release cytokines for a longer time. Some studies suggest that BCG can be alive *in vivo* for more than 20 years.

In this thesis, I will report the expression of huIL-2, mIFN-γ and huTNF-α in BCG. Moreover, we (Kong and Kunimoto, 1995) have shown that huIL-2-secreting BCG can limit the replication of BCG in BALB/c mice, which is likely due to the augmentation of the host immune response by secreted huIL-2, although further experiments are needed to identify the mechanisms involved.

In addition, as a preliminary project to learn cytokine bioassays and recombinant expression techniques, I performed *in vitro* mutagenesis on two N-glycosylation sites of mouse interleukin 5 (mIL-5) to make it more homogenous for analysis of the mIL-5 spatial structure by X-ray diffraction. Introduction to the protein, gene and function of IL-5 is presented in Chapter IV.

4. Bibliography

Adams, D.A., and T.A. Hamilton. 1984. The cell biology of macrophage activation. Annu. Rev. Immunol. 2: 283-318.

Aldovini, A., and R.A. Young. 1990. Development of a BCG recombinant vehicle for candidate AIDS vaccines. Intern. Rev. Immunol. 7: 79-83.

Aldovini, A., and R.A. Young. 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature 351: 479-482.

Anderson P.M., D.C. Hanson, D.E. Hasz, M.R. Halet, B.R. Blazar, and A.C. Ochoa. 1994. Cytokines in liposomes: preliminary studies with IL-1. IL-2, IL-6, GM-CSF and interferon-gamma. Cytokines 6: 92-101.

Andersen, A.B., A. Worsaae, and S.D. Chaparas. 1988. Isolation and characterization of recombinant lambda gt11 bacteriophages expressing eight different mycobacterial antigens of potential immunological relevance. Infect. Immun. 56: 1344-51.

Andersen, A.B., and E.B. Hansen. 1989. Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*. Infect. Immun. 57: 2481-2488.

Andrew, P.W., and D.B. Lowrie. 1988. Role of interferon in immunity to mycobacteria. In Interferon and nonviral pathogens. Edited by

Ryrne, G. I. and J. Turco. Marcel Dekker, Inc. New York. pp 263-286.

Augustin, A., R.T. Kubo, and G.K. Sim. 1989. Resident pulmonary lymphocytes expressing the gamma/delta T cell receptor. Nature (London) 340: 239-241.

Baird, P.N., L.M.C. Hall, and A.R.M. Coates. 1989. Cloning and sequence analysis of the 10 Kda antigen gene of *Mycobacterium tuberculosis*. J. Gen. Microbiol. 135: 931-9.

Balasubramanian, V., E.H. Wiegeshans, B.T. Taylor, and D.W. Smith. 1994. Pathogenesis of tuberculosis: Pathway to apical localization. Tubercle and Lung Disease 75: 168-178.

Barnes, P.F., A.B. Bloch, P.T. Davidson, and D.E. Snider, Jr. 1991. Tuberculosis in patients with human immunodeficiency virus infection. N. Engl. J. Med. 324: 1644-1650.

Barnes, P.F., D. Chatterjee, J.S. Abrams, S. Lu, E. Wang, M. Yamamura, P.J. Brennan, and R.L. Modlin. 1992. Cytokines production induced by *Mycobacterium tuberculosis* lipoarabinomannan: relationship to chemical structure. J. Immunol. 307: 1593-1597.

Barnes, P.F., R.L. Modlin, and J.J. Ellner. 1994. T cell responses and Cytokines. In Tuberculosis, Pathogenesis, Protection, and Pontrol. Edited by B.R. Bloom. ASM Press, Washington, DC. pp 417-435.

Barnes, P.F., S.-J. Fong, P. Brennan, P.E. Twomey, A. Mazumder, and R.L. Modlin. 1990. Local production of tumor necrosis factor and IFN-γ in tuberculous pleuritis. J. Immunol. 145: 149-154.

Beaman, M.H., S.Y. Wong, and J.S. Remington. 1992. Cytokines, toxoplasma and intracellular parasitism. Immunol. Rev. 127: 97-117.

Bercovier, H., O. Kafri, and S. Sela. 1986. Mycobacteria possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochem. Biophys. Res. Commun. 136: 1136-41.

Bercovier, H., O. Kafri, D. Kornitzer, and S. Sela. 1989. Cloning and restriction analysis of ribosomal RNA genes from *Mycobacterium smegmatis*. FEMS Microbiology Letters 57: 125-128.

Bermudez, L.E.M., and L.S. Young. 1988. Tumor necrosis factor alone or in combination with IL-2, but not IFN-γ, is associated with macrophage killing of *Mycobacterium avium* complex. J. Immunol. 140: 3006-3013.

Bermudez, L.E.M., P. Stevens, P. Kolonoski, M. Wu, and L.S. Young. 1989. Treatment of experimental disseminated *Mycobacterium-avium* complex infection in mice with recombinant IL-2 and tumor necrosis factor. J. Immunol. 143: 2996-3000.

Beutler, B., and A. Cerami. 1988. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. Annu. Rev. Biochem. 57: 505-18.

Bishop, P.J., and G. Neumann. 1970. The history of the Ziehl-Neelsen stain. Tubercle 51: 196-206.

Bloom, B.R. 1986. Learning from leprosy: a perspective on immunology and the third world. J. Immunol. 137: 1-10.

Bohle, A., C.H. Nowc, A.J. Ulmer, J. Musehold, J. Gerdes, A.G. Hofstetter, and H.D. Flad. 1990. Elevations of cytokines interleukin-1, interleukin-2 and tumor necrosis factor in the urine of patients after intravesical bacillus Calmette-Guérin immunotherapy. J. Urol. 144: 59-64.

Bohle, A., J. Gerdes, A.J. Ulmer, A.G. Hofstetter, and H.D. Flad. 1990. Effect of local bacillus Calmette-Guérin therapy in patients with bladder carcinoma on immunocompetent cells of the bladder wall. J. Urol. 144: 53-58.

Bradley, S.G. 1973. Relationships among mycobacteria and nocardiae based upon deoxyribonucleic acid reassociation. J. of Bacteriol. 113: 645-651.

Buchmeier, N.A., and F. Heffron. 1990. Induction of salmonella stress proteins upon infection of macrophages. Science 248: 730-732.

Burgess, R.R., B. Erickson, D. Gentry, M. Gribsker, D. Hager, S. Lesley, M. Strickland, and N. Thompson. 1987. Bacterial RNA polymerase subunits and genes. In: RNA polymerase and the regulation of transcription, Edited by Reznikoff, W.S., R.R. Burgess, J.E. Dahlberg, C.A. Gross, M.T. Record Jr. and M.P. Wickens. Elsevier, New York. pp 3-15.

Burke, M.C., A. Miyajima, and R. Coffman. 1993. T cell-derived cytokines and their receptors. In: Fundamental Immunology. Edited by Paul, W.E., Raven Press, Ltd, New York. pp 763-800.

Burlein, L., C.K. Stover, S. Offutt, and M.S. Hanson. 1994. Expression of foreign genes In: Tuberculosis, Pathogenesis, Protection, and Control, Edited by Bloom, B.R. ASM Press, Washington. pp239-252.

Calmette, A., C. Guérin, and B. Weill-Halle. 1924. Essais de premunition par le BCG contre l'infection tuberculoses de l'homme et des animaux. Bull.Acad.Natl.Med.(Paris) 91: 787-796.

Cassatt, D.R., U.F. de la Cruz, J.E. Burlein, J. Young, S. Koenig, and C.K. Stover. 1993. Protection of mice against tetanus challenge using an experimental tetanus vaccine based on recombinant BCG. In Vaccines, edited by H.S. Ginsberg, F. Brown, R.M. Channock, and R.A. Lerner, Cold Spring Harbor Laboratory Press, Plainview, New York. pp385-389.

Chan, J., and S.H.E. Kaufmann. 1994. Immune mechanisms of protection. In Tuberculosis, Pathogenesis, Protection, and Control. Edited by B.R. Bloom. ASM Press, Washington, DC. pp 389-415.

Chensue, S., I. Otterness, G. Higashi, C. Forsch, and S. Kunkel. 1989. Monokine production by hypersensitivity (*Schistosoma mansoni* egg) and foreign body (sephadex bead)-type granuloma macrophages. Evidence for sequential production of IL-1 and tumor necrosis factor. J. Immunol. 142: 1281-86.

Chun, M., M. Krim, and A. Granelli-Piperno. 1985. Enhancement of cytotoxic activity of natural killer cells by interleukin 2, and antagonism between interleukin 2 and adenosine cyclic monophosphate. Sand. J. Immunol. 22: 375-81.

Clark-Curtiss, J.E. 1990. Genome structure of mycobacteria. In: Molecular Biology of Mycobacteria, Edited by MaFadden, Academic Press Ltd, London. pp77-96.

Clark-Curtiss, J.E., W.R. Jacobs, M.A. Docherty, L. R. Ritchie, and R. Curtiss. 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae* DNA. J. Infect. Dis. 159: 7-15.

Clark-Curtiss, J.E., W.R. Jacobs, M.A. Docherty, L.R. Ritchie, and R. Curtiss III. 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*. J. Bacteriol. 161: 1093-1120.

Cole, S.T., and D.R. Smith. 1994. Toward mapping and sequencing the genome of *Mycobacterium tuberculosis*. In: Tuberculosis, Pathogenesis, Protection, and Control, Edited by Bloom, B.R. ASM Press, Washington. pp227-238.

Collins, M., M.T., Moss, S. Wall, and J. W. Dale. 1987. Expression of biotinylated proteins in mycobacterial gene libraries. FEMS microbiol. Lett. 43: 53-56.

Colston, M.J., and E.O. Davis. 1994. Homologous recombination, DNA repair, and mycobacteria recA genes, In: Tuberculosis,

Pathogenesis, Protection, and Control, Edited by Bloom, B.R. ASM Press, Washington. pp217-226.

Connell, N.D., E. Medina-Acosta, W.R. McMaster, B.R. Bloom, and D.G. Russell. 1993. Effective immunization against cutaneous leishmaniasis with recombinant Bacille Calmette-Guérin expressing the leishmania surface proteinase gp63. Proc. Natl. Acad. Sci. USA 90: 11473-11477.

Copper, A.M., D.K. Dalton, T.A. Stewart, J.P. Griffin, D.G. Russell, and I.M. Orme. 1993. Disseminated tuberculosis in interferon-γ gene-disrupted mice. J. Exp. Med. 178: 2243-2247.

Crawford, J.T., and J.H. Bates. 1979. Isolation of plasmids from mycobacteria. Infect. Immun. 24: 979-981.

Crowle, A.J., E.J. Ross, and M.H. May. 1987. Inhibition by 1, 25 (OH)2-vitamin D3 of the multiplication of virulent tubercle bacilli in cultured human macrophages. Infect. Immun. 55: 2945-2950.

Dale, J.W., and A. Patki. 1990. Mycobacterial gene expression and regulation. In: Molecular Biology of mycobacteria, Edited by McFadden, Academic Press Ltd, London pp173-198.

Davis, M.M., and P.J. Bjorkman. 1988. T cell antigen receptor genes and T cell recognition. Nature (London) 334: 395-402.

De Boer E.C., W.H. De Jong, A.P.M. Van der Meijden, P.A. Steerenberg, F. Witjes, P.D.J. Vegt, F.M.G. Debruyne, and E.J. Ruitenberg. 1991. Leukocytes in the urine after intravesical BCG treatment for

superficial bladder cancer. A flow cytofluorometric analysis. Urol. Res. 19: 45-50.

de la Hera, A., M.L. Toribio, C. Marquez, and A.C. Martinez. 1985. Interleukin 2 promotes growth and cytolytic activity in human $T3^{+}4^{-}8^{-}$ thymocytes. Proc. Natl. Acad. Sci. USA 82: 6268-71.

DeLibew, G., I. Flesch, and S.H.E. Kaufmann. 1988. Mycobacterial reactive $lyt2^{+}$ T cells lines. Eur. J. Immunol. 18: 59-66.

Dellagostin, O., S. Wall, E. Norman, T. O'Shaughnessy, J.W. Dale, and J.J. McFadden. 1993. Constuction and use of integrative vectors to express foreign genes in mycobacteria. Molecular Microbiology 10: 983-993.

Denis, M. 1991. Involvement of cytokines in determining resistance and acquired immunity in murine tuberculosis. J. Leuk. Biol. 50: 495-501.

Denis, M. 1991. Tumor necrosis factor and granulocyte-macrophage colony stimulating factor stimulate human macrophages to restrict growth of virulent *Mycobacterium avium* and to kill avirulent *M. avium*: killing effector mechanism depends on the generation of reactive nitrogen intermediates. J. Leukocyte Biol. 49. 380-387.

Dinarello, C.A., J.G. Cannon, and S.M. Wolff. 1986. Tumor necrosis factor (Cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med. 163: 1433-1450.

Ding, A.H., C.F. Nathan, and D.J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from

mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. J. Immunol. 141: 2407-2412.

Doi, R.H. 1991. Regulation of gene expression In: Modern microbial genetics, Edited by Streips, U.N. and R.E. Yasbin, Wiley-Liss, Inc. (New York) pp15-39.

Doi, R.H., and L.F. Wang. 1986. Multiple prokaryotic ribonucleic acid polymerase sigma factors. Microbial. Rev 50: 227-243.

Donnelly-Wu, M.K., W.R. Jacobs, Jr., and G.F. Hatfull. 1993. Superinfection immunity of mycobacteriophage L5: applications for genetic transformation of mycobacteria. Mol. Microbiol. 7: 407-417.

Douvas, G.S., D.L. Looker, A.E. Vatter, and A.J. Crowle. 1985. Gamma interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria. Infect. Immun. 50: 1-8.

Draper, P. 1982. The anatomy of mycobacteria in The Biology of the mycobacteria. Edited by Ratledge, C. and J. Stanford. Academic Press (London). pp 9-52.

Durum, S.K., and J.J. Oppenheim. 1993. Proinflammatory cytokines and immunity. In: Fundamental Immunology, edited by Paul, W.E. Raven Press, Ltd., New York. pp801-835.

Eiglmeier, K., N. Honore, S.A. Woods, B. Canldron, and S.T. Cole. 1993. Use of an ordered cosmid library to deduce the genomic organization of *Mycobacterium leprae*. Mol. Microbiol. 7: 197-206.

El-demiry, M.I.M., G. Smith, A.W.S. Ritchie, K. James, J.A. Cumming, T.B. Hargreave, and G.D. Chisholm. 1987. Local immune responses after intravesical BCG treatment for carcinoma *in situ*. Br. J. Urol. 60: 543-548.

Esparza, I., D. Mannel, A. Ruppel, W. Falk, and P.H. Krammer. 1987. Interferon γ and lymphotoxin or tumor necrosis factor act synergistically to induce macrophage killing of tumor cells and achistosomula of *Schistosoma mansoni*. J. Exp. Med. 166: 589-594.

Falkinham III, J.O., and J.T. Crawford. 1994. Plasmid. In: Tuberculosis, Pathogenesis, Protection, and Control, Edited by Bloom, B.R. ASM Press, Washington. pp185-198.

Farrar, M.A., and R.D. Schreiber. 1993. The molecular cell biology of interferon-γ and its receptor. Ann. Rev. Immunol. 11: 571-611.

Fiers, W. 1991. Tumor necrosis factor, characterization at the molecular, cellular and *in vivo* level. FEBS Letters 285: 199-212.

Fiesch, I.E.A., and S.H.E. Kaufmann. 1990. Activation of tuberculostatic macrophage functions by interferon-γ, interleukin-4 and tumor necrosis factor. Infect. Immun. 58: 2675-2677.

Filley, E.A., and G.A.W. Rook. 1991. Effect of mycobacteria on sensitivity to cytotoxic effects of tumor necrosis factor. Infect. Immun. 59: 2567-2572.

Fine, P.E. 1989. The BCG story: lessons from the past and implications for the future. Rev. Infect. Dis. 11 (Suppl. 2): 253-359.

Flesch, I.E.A., and S.H.E. Kaufmann. 1993. Role of cytokines in tuberculosis. Immunobiol. 189: 316-339.

Flynn, J.L., J. Chan, K.J. Triebold, D.K. Dalton, T.A. Stewart, and B.R. Bloom. 1993. An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. 178: 2249-2254.

Flynn, J.L., M.A. Goldstein, K.J. Treibold, B. Kollerand, and B.R. Bloom. 1992. Major histocompatibility complex class-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc. Natl. Acad. Sci. USA 89: 12013-12017.

Fortier, A.H., B.A. Mock, M.S. Meltzer, and C.A. Nacy 1987. *Mycobacterium bovis* BCG-induced protection against cutaneous and systemic *Leishmania major* infection of mice. Infect. immun. 55: 1707-1714.

Gajewski, T.F., S.R. Schell, G. Nau, and F.W. Fitch. 1989. Regulation of T cell activation: differences among T cell subsets. Immunol. Rev. 111: 79-10.

Gardner, G.M., and R.S. Weiser. 1947. A bacteriophage for *Mycobacterium smegmatis*. Proc. Soc. Exp. Biol. Med. 66: 205-206.

Germain, R.N. 1993. Antigen processing and presentation. In Fundamental immunology. Third edition, Edited by W.E. Paul. Raven press, Ltd., New York pp629-676.

Gillis, S., N.A. Union, P.E. Baker, and K.A. Smith. 1979. The *in vitro* generation and sustained culture of nude mouse cytolytic T-lymphocytes. J. Exp. Med. 149: 1460-1466.

Gottesman, S. 1984. Bacterial regulation: Global regulatory networks. Ann. Rev. Genet. 18: 425-41.

Grange, J.M. 1988. Leprosy in Mycobacteria and Human Disease. Edward Arnold Ltd. (London). pp 102-118

Grange, J.M. 1988. The genus Mycobacterium. In Mycobacteria and Human Disease. Edward Arnold Ltd. (London). pp 9-30.

Grange, J.M. 1988. The species of Mycobacterium. In Mycobacteria and Human Disease. Edward Arnold Ltd. (London). pp 31-48.

Grange, J.M. 1988. Tuberculosis. In Mycobacteria and Human Disease. Edward Arnold Ltd. (London). pp 117-137.

Gray, P.W., and D.V. Goeddel. 1983. Cloning and expression of murine immune interferon cDNA. Proc. Natl. Acad. Sci. USA 80: 5842-46.

Gray, P.W., D.W. Leung, D. Pennica, E. Yelverton, R. Najarian, C.C. Simonsen, R. Derynck, P.J. Sherwood, D.M.Wallace, S.L. Berger, A.D. Levinson, and D.V. Goeddel. 1982. Expression of human immune interferon cDNA in *E. coli* and monkey cells. Nature 295: 503-8.

Greenlund, A.C., C.A. Wenner, and R.D. Schreiber. 1992. Analysis of IFN-γ receptor binding to IFN-γ. J. Interferon Res. (Abstr.) 12: S103.

Grimald, G.F., P. Moriearty, and R. Hoff. 1980. *Leishmania mexicana* in C3H mice: BCG and levamisole treatment of established infections. Clin. Exp. Immunol. 41: 237-242.

Grossman, A.D., J.W. Erickson, and C.A. Gross. 1984. The htpR gene product of *E. coli* is a sigma factor for heat-shock promoters. Cell 38: 383-390.

Groves, M.J. 1993. Pharmaceutical characterization of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccine used for the treatment of superficial bladder cancer. J. of Pharmaceutical. Sciences 82: 555-562.

Harboe, M., S. Nagai, M.E. Patarroyo, M.L. Torres, C. Ramirez, and N. Cruz. 1986. Properties of proteins MPB64, MPB70, and MPB80 of *Mycobacterium bovis* BCG. Infect. Immun. 52: 293-302.

Hatfull, G.F., and W.R. Jacobs, Jr. 1994. Mycobacteriophages: cornerstones of mycobacterial research. In: Tuberculosis, Pathogenesis, Protection, and Control, Edited by Bloom, B.R. ASM Press, Washington. pp165-183.

Herr, H.W., and W.F. Whitmore. 1987. Ureteral carcinoma in situ after successful intravescal therapy for superficial bladder tumors. J. Urol. 138: 292-294.

Hirschman, J. P., K. Wong, K. Sei, J. Keener, and S. Kustu. 1985. Products of nitrogen regulatory genes ntrA and ntrC of enteric bacteria activate glnA transcription *in vitro*: evidence that the

ntrA product is a sigma factor. Proc. Natl. Acad. Sci. USA 82: 7525-7529.

Hocker S., G. Franke, I. Paulini, and M. L. Lohmann-Matthes. 1991. Immunotherapy of murine visceral leishmaniasis with murine recombinant interferon-gamma and MTP-PE encapsulated in liposomes. J. Interferon Res. 11: 177-185.

Honore, N., S. Chanteau, F. Doucet-Populaire, K. Eiglmeier, T. Garnier, C. Georges, P. Launois, P. Limpaiboon, S. Newton, K. Nyang, P. del Portillo, G.K. Ramesh, T. Reddy, J.P. Riedel, N. Sittissombut, S. WunHunter, and S.T. Cole. 1993. Nucleotide sequence of the first cosmid from the *Mycobacterium leprae* genome project: structure and function of the kif-str regions. Mol. Microbiol. 7: 207-214.

Hopewell, P.C. 1994. Overview of clinical tuberculosis. In Tuberculosis, Pathogenesis, Protection, and Control. Edited by B.R. Bloom. ASM Press, Washington, DC. pp25-46.

Horsburgh Jr, C.R. 1991. *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. N. Engl. J. Med 324: 1332-1338.

Hunt, T.P., and B. Magasanlk. 1985. Transcription of glnA by purified *Escherichia coli* components: Core RNA polymerase and the products of gluF, gluG, and gluL. Proc. Natl. Acad. Sci. USA 82: 8453-8457.

Husson, R.N., B.E. James, and R.A. Young. 1990. Gene replacement and expression of foreign DNA in mycobacteria. J. Bacteriol. 172: 519-524.

Imaeda, T., W.F. Kirchheimer, and L. Barkadale. 1982. DNA isolated from *Mycobacterium leprae*: Genome size, base ratio, and homology with other related bacteria as determined by optical DNA-RNA reassociation. J. Bacteriol. 150:414-417.

Izzo, A.A., and R.J. North. 1992. Evidence for an α/β T cell independent mechanism of resistance to mycobacteria Bacillus-Calmette-Guérin causes progressive infection in severe combined immunodeficient mice, but not in nude mice or mice depleted of $CD4^+$ and $CD8^+$ T cells. J. Exp. Med. 176: 581-586.

Jacobs Jr., W.R., M. Tuckman, and B.R. Bloom. 1987. Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature (London) 327: 532-535.

Jacobs, W.R., J.F. Barrett, J.E. Clark-Curtiss, and R. CurtissIII. 1986. *In vitro* repackaging of recombinant cosmid molecules for analysis of *Salmonella typhimurium*, *Streptococcus mutans*, and mycobacterial genomic libraries. Infect. Immun. 52: 101-109.

Jacobs, W.R., Jr., G.V. Kalpana, J.D. Cirillo, L. Pascopella, S.B. Snapper, R.A. Udani, W. Jones, R.G. Barletta, and B.R. Bloom. 1991. Genetic systems for mycobacteria. Methods Enzymol. 204: 537-555.

Jacobs, W.R., Jr., M. Tuckman, and B.R. Bloom. 1987. Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature (London) 327: 532-535.

Jacobs, W.R., M.A. Docherty, R. Curtiss III, and J.E. Clark-Curtiss. 1986. Expression of *Mycobacterium leprae* genes from a *Streptococcus mutans* promoter in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA 83:1925-1930.

Janis, E.M., S.H.E. Kaufmann, R.H. Schwartz, and D.M. Pardoll. 1989. Activation of γδ T cells in the primary immune response to *Mycobacterium tuberculosis*. Science 244: 713-716.

Jeevan, A., and G.L. Asherson. 1988. Recombinant interleukin-2 limits the replication of *Mycobacterium lepraemurium* and *Mycobacterium bovis* BCG in mice. Infect. Immun. 56: 660-664.

Jones, E. Y., D.I. Stuart, and N.P.C. Walker. 1989. Structure of tumor necrosis factor. Nature 338: 225-228.

Jopling, W.H. 1984. Handbook of Leprosy, 3rd edition, London: William Heinemann.

Kaufmann, S.H.E. 1993. Immunity to intracellular bacteria. In Fundamental Immunology. Third edition Edited by W.E. Paul. Raven press, Ltd., New York pp1251-1286.

Kavoussi, L.R., E.J. Brown, J.K. Ritchey, T.L.J. Ratliff. 1990. Fibronectin-mediated Calmette-Guérin bacillus attachment to murine baldder mucosa requirement for the expression of an antitumor response. Clinic Invest. 85: 62-67.

Kawakami, M., P.H. Pekala, M.D. Lane, and A. Cerami. 1982. Lipoprotein lipase suppression in 3T3-Ll cells by an endotoxin-induced mediator from exude cells. Proc. Natl. Acad. Sci. USA 79: 912-916.

Kindler, V., A.-P. Sappino, G.E. Gary, P.-F. Pignet, and P. Vassalli. 1989. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell 56: 731-740.

Kirstein, M., and C. Baglioni. 1986. Tumor necrosis factor induces synthesis of two proteins in human fibroblasts. J. Biol. Chem. 261: 9565-9567.

Labidi, A. E., Mardis, B.A. Roe, and R.J. Wallace, Jr. 1992. Cloning and DNA sequence of the *Mycobacterium fortuitum* plasmid pAL5000. Plasmid 27:130-140.

Labidi, A., C. Dauget, K.S. Goh, and H.L. David. 1984. Plasmid profiles of *Mycobacterium fortuitum* complex isolates. Curr. Microbiol. 11: 235-240.

Lakhanpal, S., N.J. Gonchoroff, and B.S. Handwerger. 1987. Interleukin 2 induces proliferation of normal "resting" human T cells in the absence of other known external stimulation. Cell Immunol. 106: 1550-1556.

Langer, J.A., and Pestka, S. 1985. Structure of interferons. Pharmc. Ther. 27: 371-401.

Leong, AS-Y., P. Wannakrairoy, J. Jose, and J. Milios. 1990. Bacillus Calmette-Guérin treated superficial bladder cancer: correlation of morphology with immunophenotyping. J. Pathol. 162: 35-41.

Levin, M.E., and G.F. Graham. 1993. *Mycobacterium smegmatis* RNA polymerase: DNA supercoiling, action of rifampicin and mechanism of rifampicin resistance. Molecular Microbiology 8: 277-285.

Lewin, B. 1994. Control of prokaryotic gene expression. In Genes V, John Wiley and Sons, Inc. (New York) pp377-489.

Liew, F. Y., and F.E.G. Cox. 1991. Nonspecific defense mechanism: the role of nitric oxide. Immunol. Today 12A: 17-21.

Liew, R.Y., Y. Li, and S. Millott. 1990. Tumor necrosis factor-α synergizes with IFN-γ in mediating killing of *Leishmania major* through the induction of nitric oxide. J. Immunol. 145: 4306.

Lotte, A., O. Wasz-Hockert, N. Poisson, N. Dumitrescu, M. Verron and E. Couvet. 1984. BCG complications. Adv. Tuberc. Res. 21: 107-193.

Lotze, M.T., and S.A. Rosenberg. 1988. Interleukin-2 as a pharmacologic reagent. In: interleukin 2, edited by Smith, K.A. Academic Press, San Diego pp237-294.

Lugosi, L. 1992. Theoretical and methodological aspects of BCG vaccine from the discovery of Calmette and Guérin to molecular biology: A view. Tubercle and Lung Disease 73: 252-261.

Lumpkin. L.R., G.F. Cox, and J.E. Wolf. 1983. Leprosy in five armadillo handlers. J. Ame. acad. of Dermat. 9: 899-903.

Martin, C., J. Timm, J. Rauzier, R. Gomez-Lus, J. Davies, and B. Gicquel. 1990. Transposition of an antibiotic resistance element in mycobacteria. Nature (London) 345: 739-743.

Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, and T. Yamada. 1988. Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular α antigen. J. bacteriol. 170: 3847-3854.

Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, T. Terasake, M. Totsuka, K. Kobayashi, H. Yukitake, and T. Yamada. 1990. Establishment of a foreign secretion system in mycobacteria. Infect. Immun. 58: 4049-4054.

Mbawuike, I.N., P.R. Wyde, P.M. Anderson. 1990. Enhancement of the protective efficacy of inactivted influenza A virus vaccine in aged mice by IL-2-liposomes. Vaccine 8: 347-352.

McFadden, J. 1990. Molecular Biology of Mycobacteria. Academic press Ltd, London.

McFadden, J.J., P.D. Butcher, R.J. Chiodin, and J. Hermon-Taylor. 1987. Determination of genome size and DNA homology between an unclassified mycobacterium species isolated from patients with Crohn's disease and other mycobacteria. J. Gen Microbiol. 133: 211-214.

Mehra, V., B.R. Bloom, A.C. Bajardi, C.L. Grisso, P.A. Sieling, D. Alland, J. Convit, X. Fan, S.W. Hunter, P.J. Brennan, T.H. Rea, and R.L. Modlin. 1992. A major T cell antigen of *Mycobacterium leprae* is a 10-KD heat-shock cognate protein. J. Exp. Med. 175: 275-284.

Mehra, V., D. Sweetser, and R.A. Young. 1986. Efficient mapping of protein antigenic determinants. Proc. Natl. Acad. Sci. USA 83: 7013-7017.

Minami, Y., T. Kono, T. Miyazaki, and T. Taniguchi. 1993. The IL-2 receptor complex: its structure, function, and target genes. Ann. Rev. Immunol. 11: 245-67.

Modlin, R.L. 1994. Th1-Th2 paradigm: Insights from leprosy. J. Invest. Dermotol. 102: 828-832.

Mombaerts, P., A.R. Clarke, M.A. Rudnicki, J. Lacomin, S. Itohara, J.J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M. L. Hooper and S. Tonegawa. 1991. Mutations in T cell antigen receptor genes α and β block thymocytes development at different stages. Nature (London) 360: 225-231.

Mombaerts, P., J. Arnold, F. Russ, S. Tonegawa, and S.H.E. Kaufmann. 1993. Differential roles of α/β and γ/δ T cells in immunity against an intracellular bacterial pathogen. Nature (London) 365:52-56.

Moreno, C.J., J. Taverne, A. Mehlert, C.A.W. Bath, R.J. Brealey, A. Meager, G.A.W. Rook, and J.H.L. Playfair. 1989. Lipoarabinomannan from *Mycobacterium tuberculosis* induces the production of tumor necrosis factor from human and murine macrophages. Clin. Exp. Immunol. 76: 240-245.

Morgan, D.A., F.W. Ruscett, and R.C. Gallo. 1976. Selection *in vivo* growth of T lymphocytes from normal human bone marrows. Science 193: 1007-1008.

Mosmann, T.R., and R.L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev. Immunol. 7: 145-173.

Mulkerrin, M.G., and R. Wetzel. 1989. pH dependence of the reversible and irreversible thermal denaturation of gamma interferons. Biochemistry 28: 6556-61.

Muller, I, S.P. Cobbold, H. Waldmann, and S.H.E. Kaufmann. 1987. Impaired resistance against *Mycobacterium tuberculosis* infection after selective *in vivo* depletion of L3T4$^+$ and lyt2$^+$ T cells. Infect. Immun. 55: 2307-2041.

Munro, J.M., J.S. Pober, and R.S. Cotran. 1989. Tumor necrosis factor and interferon-gamma induce distinct patterns of endothelial activation and associated leukocyte accumulation in skin of Papio-Anubis. Am. J. Pathol. 135: 121-33.

Murry, A., N. Winter, M. Lagranderie, D.F. Hill, J. Rauzier, J. Timm, C. Leclerc, K.M. Moriarty, M. Gheorghiu, and B. Gicquel. 1992. Expression of *Escherichia coli* β-galactosidase in *Mycobacterium bovis* BCG using an expression system from *Mycobacterium paratuberculosis* which induced humoral and cellular immune responses. Mol. Microbiol. 6: 3331-3342.

Murry, H.W., G.L. Spitalny, and C.F. Nathan. 1985. Activation of mouse peritoneal macrophages *in vitro* and *in vivo* by interferon γ. J. Immunol. 134: 1619.

Nagata, K., T. Izumi, T. Kitagawa, and N. Yoshida. 1987. Oligomeric structure of recombinant human and murine immune interferons by means of sedimentation equilibrium. J. Interferon Res. 7: 313-20.

Nathan, C. 1992. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6: 3051-3064.

Ogawa, T., H. Uchida, Y. Kusomoto, Y. Mori, Y. Yamamura, and S. Hamada. 1991. Increase in tumor necrosis factor-α and interleukin-6-secreting cells in peripheral blood mononuclear cells from subjects infected with *Mycobacterium tuberculosis*. Infect. Immun. 59: 3021-3025.

Old, L.J. 1985. Tumor necrosis factor (TNF). Science 230: 639-32.

Orme, I.M., and F.M. Collins. 1983. Protection against *Mycobacterium tuberculosis* infection by adoptive immunotherapy. J. Exp. Med. 158: 74-83.

Orme, I.M., and F.M. Collins. 1984. Adoptive protection of the *Mycobacterium tuberculosis* infected lung. Dissociation between cells and those that passively transfer protective immunity and those that transfer delayed type hypersensitivity to tuberculin. Cell. Immunol. 84: 113-120.

Orme., I.M. 1987. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with *Mycobacterium tuberculosis*. J. Immunol. 138: 293-298.

Pace, J.L., S.W. Russell, B.A. Torres, H.M. Johnson, and P.W. Gray. 1983. Recombinant mouse γ interferon induces the priming step in macrophage activation for tumor cell killing. J. Immunol. 130: 2011-2013.

Padgett, E.L., and S.B. Bruett. 1992. Evaluation of nitrite production by human monocyte-derived macrophages. Biochem. Biophy. Res. Commun. 186: 775-781.

Pedrazzin, T., K. Hug, and J. A. Louis. 1987. Importance of L3T4$^+$ and lyt2$^+$ cells in the immunologic control of infection with *Mycobacterium bovis* strain bacillus Calmette-Guérin in mice: Assessment by elimination of T cells subsets *in vivo*. J. Immunol. 139:2032-2037.

Pennica, D., G.E. Nedwin, and J.S. Hayflick. 1984. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 312: 724-729.

Predich, M., L. Doukhan, G. Nair, and I. Smith. 1995. Characterization of RNA polymerase and sigma-factor genes from *Mycobacterium smegmatis*. Molecular Microbiology 15: 355-366.

Prescott, S., K. James, T.B. Hargreave, G.D. Chisholm, and J.F. Smyth. 1990. Radioimmunoassay detection of interferon-gamma in urine after intravesical evans BCG therapy. J. Urol. 144: 1248-1251.

Quinn, T. C. 1989. Interactions of the human immunodeficiency virus and tuberculosis and the implications for BCG vaccination. Rev. Infect. Dis. 11: S379-S384.

Radford, A.J., and A.L.M. Hodgson. 1991. Construction and characterization of a mycobacterium-*Escherichia coli* shuttle vector. Plasmid 25: 149-153.

Rajala, P., E. Kaasinen, E. Rintala, K. Jauhiainen, M. Nurmi, O. Alfthan, and M. Lahde. 1992. Cytostatic effect of different strains of bacillus Calmette-Guérin on human bladder cancer cells *in vitro* alone and in combinantion with mitomycin C and interferon-α. Urol. Res. 20: 215-217.

Ratliff, T.L., D.P. Gillen, and W.J. Catalona. 1987. Requirement of a thymus dependent immune response for BCG-mediated antitumor activity. J. Urol. 137: 155-157.

Ratliff, T.L., J.O. Palmer, and J.A. MaGan. 1987. Intravesical BCG therapy for murine bladder tumors. Initiation of response by fibronectin mediated attachment of BCG. Cancer Res. 47: 1762-1766.

Raulet, D.H. 1989. The structure, function, and molecular genetics of the γ/δ T receptor. Ann. Rev. Immunol. 7: 175-207.

Reznikoff, W.S., D.A. Siegele, D.W. Cowing, and C.A. Gross. 1985. The regulation of transcription initiation in bacteria. Ann. Rev. Genet. 19: 355-87.

Ridley, D.S., and W.H. Jopling. 1966. Classification of leprosy according to immunity: A five-group system. Int. Lepr. 34: 255-273.

Rook, G. A. W. 1988. The role of vitamin D in tuberculosis. Am. Rev. Respir. Dis. 138: 768-770.

Rook, G.A.W., J. Sreele, M. Ainsworth, and B.R. Champion. 1986. Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: Comparison of the effects of recombinant gamma interferon on human monocyte and murine peritoneal macrophages. Immunology 59: 333-338.

Rook, G.A.W., J. Taverne, C. Leveton, and J. Steele. 1987. Th role of gamma-interferon, vitamin D3 metabolites and tumor necrosis factor in the pathogenesis of tuberculosis. Immunology 62: 229-34.

Rosenberg, S.A., and M.T. Lotze. 1986. Cancer immunotherapy using interleukin-2 and interleukin-2-activated lymphocytes. Ann. Rev. Immunol. 4: 681-709.

Savary, C.A., E. Lotzova, and J. Klostergaard. 1989. Interleukin 2-activated large granular lymphocytes: cytotoxic efficiency and mechanism of killing of tumor cell lines. Immunol. Lett. 21: 145-51.

Sbarbaro, J.A. 1986. Skin testing in the diagnosis of tuberculosis. Semin. Respir. Infect 1: 234-238.

Scahill, S.J., R. Devos, J. Van der Heyden, and W. Fiers. 1983. Expression and characterization of the product of a human immune interferon cDNA gene in Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA 80: 4654-58.

Sela, S., J.E. Clark-Curtiss, and H. Bercovier. 1989. Characterization and taxonomic implications of the rRNA genes of *Mycobacterium leprae*. J. of Bacteriol. 171: 70-73.

Sheppard, C.C. 1958. A comparison of the growth of selected mycobacteria in Hela, monkey kidney, and human amnion cells in tissue culture. J. Exp. Med. 107: 237-245.

Sher, A., and R.L. Coffman. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. Ann. Rev. Immunol. 10: 385-409.

Sher, N.A., S.D. Chaparas, L.F. Greenberg, E.M. Merchant, and J. H. Vickers. 1975. Response of congenitally athymic (nude) mice to infection with *Mycobacterium bovis* (Strain BCG). J. Natl. Cancer Inst. 54: 1419-1426.

Sherman, M.P., M.L. Loro, V.Z. Wong, and D.P. Tashkin. Cytokine- and *Pneumocystis carinii*-induced L-arginine oxidation by murine and human pulmonary alveolar macrophages. J. Protozool. 38: 2345-2365.

Shinnick, T.M. 1987. The 65-Kilodalton antigen of *Mycobacterium tuberculosis*. J. Bacteriol. 169: 1080-1088.

Smith, K.A. 1988. Interleukin-2: inception, impact, and implications. Science 240: 1169-1176.

Smith, R.A., and C. Baglioni. 1987. The active form of tumor necrosis factor is a trimer. J. Biol. Chem. 262: 6951-6954.

Snapper, S.B., L. Lugosi, A. Jekkel, R.E. Melton, T. Kieser, B.R. Bloom, and W.R. Jacobs, Jr. 1988. Lysogeny and transformation in mycobacteria: stable expression of foreign genes. Proc. Natl. Acad. Sci. USA 85: 6987-6991.

Stein, M., and S. Gordon. 1991. Regulation of tumor necrosis factor (TNF) release by murine peritoneal macrophages: role of cell stimulation and specific phagocytic plasma membrane receptors. Eur. J. Immunol. 21: 431-37.

Stover, C.K., G.P. Bansal, M.S. Hanson, J.E. Burlein, S.R. Palaszynski, J.F. Young, S. Koenig, D.B. Young. A. Sadziene, and A. Barbour. 1993. Protective immunity elicited by a recombinant bacille Calmette-Guérin (BCG) expressing outer surface protein A (osp A) lipoprotein: a candidate lyme disease vaccine. J. Exp. Med. 178: 197-209.

Stover, C.K., V.F. De la Cruz, T.R. Fuerst, J.E. Burlein, L.A. Benson, L.T. Bennett, G.P. Bansal, J.F. Young, M.H. Lee, G.F. Hatfull, S.B. Snapper, R.G. Barletta, W.R. Jacobs,Jr., and B.R. Bloom. 1991. New use of BCG for recombinant vaccines. Nature 351:456-460.

Stover, V., and H. Peter. 1980. BCG immunotherapy fro prevention of relapse in patients with bladder cancer. Theropie Woche. 30: 60-64.

Styblo, K. 1989. Overview and epidemiological assessment of the current global tuberculosis situation with an emphasis on control in developing countries. Rev. Infect. Dis. 11: 339-S346.

Swain, S.L., L.M. Bradley, M. Croft, S. Tonkonogy, G. Atkins, A.D. Weinberg, D.D. Duncan, S.M. Hedrick, R.W. Dutton, and G. Huston. 1991. Helper T cell subsets: phenotype, function and the role of lymphokines in regulating their development. Immunol. Rev. 123: 115.

Taniguchi, T., H. Matsui, T. Fujita, C. Takaoka. N. Kashima, R. Yoshimoto, and J. Hamuro. 1983. Structure and expression of a cloned cDNA for human interleukin-2. Nature 302: 305-310.

Taylor, W.E., D.B. Straus, A.D. Grossman, Z.F. Burton, C.A. Gross, and R.R. Burgess. 1984. Transcription from a heat-inducible promoter causes heat shock regulation of the sigma subunit of *E. Coli* RNA polymerase. Cell 38: 371-381.

Thole, J.R., W.J. Keulen, A.H.J. Kolk, D.G. Groothuis, L.G. Berwald, R.H. Tiesjema, and van Embden. 1987. Characterization, sequence determination, and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli*. K-12. Infect. Immun. 55: 1466-1475.

Toossi, Z., T. Young, P. Gogate, and J.J. Ellner. 1991. Expression of transforming growth factor-β in lung granulomas and peripheral blood monocytes of patients with tuberculosis, Abstr., P57-62. Twenty-sixth Joint Conf. Tuberc. Lepr.

Trinchieri, G., and B. Perussia. 1985. Immune interferon: a pleiotropic lymphokine with multiple effects. Immunol. Today 6: 131-136.

Valone, S.E., E.A. Rich, and R.S. Ellner. 1988. Expression of tumor necrosis factor *in vitro* by human mononuclear phagocytes stimulated with whole *Mycobacterium bovis* BCG and mycobacterial antigens. Infect. Immun. 56: 3313-15.

Vassalli, P. 1992. The pathophysiology of tumor necrosis factors. Ann. Rev. Immunol. 10: 411-52.

Wallis, R.S., M. Amir-Tahmasseb, and J.J. Ellner. 1990. Induction of interleukin 1 and tumor necrosis factor by mycobacterial proteins: the monocyte western blot. Proc. Natl. Acad. Sci. USA 87: 3348-3352.

Watanabe, N., Y. Niitsu, H. Neda, H. Sone, N. Yamauchi, M. Maeda, and I. Urushizaki. 1988. Cytocidal mechanism of TNF: effects of lysosomal enzyme and hydroxyl radical inhibition on cytotoxicity. Immunopharmacol. Immunotoxicol. 10: 109-116.

Wayne, L. G., and G. P. Kubica. 1986. The mycobacteria. In Bergey's Manual of Systematic Bacteriology vol. 2. pp1435-1457.

Wayne, L.G., and W.M. Gross. 1968. Base composition of deoxyribonucleic acid isolated from mycobacteria. J. Bacteriol. 96: 1915-19.

Weill-Halle, B., and R. Turpinr. 1925. Primers essais de vaccination antituberculeuse del'enfant par le bacille calmette-Guérin (BCG). Bull Mem. Soc. Med. Hop Paris 49: 1589-1601.

Wheelock, E.F. 1965. Interferon-like virus-inhibitors induced in human leukocytes by phytohemogglutinin. Science 149: 310-311.

Winter, N., M. Lagranderie, J. Rauzier, J. Timm, C. Leclerc, B. Guy, M.P. Kieny, M. Gheorghin, and B. Gicquel. 1991. Expression of heterogeneous genes in *Mycobacterium bovis* BCG: induction of a cellular response against HIV-1 Nef protein. Gene 109: 47-54.

Wirth, J.J., and R. Kierszenbaum. 1988. Recombinant tumor necrosis factor enhances macrophage destruction of *Trypanosome cruzi* in the presence of bacterial endotoxin. J. Immunol. 141: 286-288.

Wong, G.H.W., and D.V. Goeddel 1988. Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. Science 242: 941-44.

Wong, G.H.W., J.H. Elwell, L.W. Oberley, and D.V. Goeddel 1989. Manganous superoxide dismutase is essential for cellular resistance to cytotoxity of tumor necrosis factor. Cell 58: 923-31.

Yamaguchi, R., K. Matsuo, A. Yamazaki, S. Nagai, K. Terasaka, and T. Yamada. 1988. Immunogenic protein MPB57 from *Mycobacterium bovis* BCG: molecular cloning, nucleotide sequence and expression. FEBS letters 240: 115-117.

Yamauchi, N., H. Kuriyama, N. Watanabe, H. Neda, M. Maeda, and Y. Niitsu. 1989. Intracellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells *in vitro*. Cancer Res. 49: 1671-1675.

Young, D., R. Lathigra, R. Hendrix, D. Sweetser, and R.A. Young. 1988. Stress proteins are immune targets in leprosy and tuberculosis. Proc. Natl. Acad. Sci. USA 85: 4267-4270.

Young, D., T., Garbe, R. Lathigra, and C. Abou-Zeid. 1990. Protein antigens: structure, function and regulation. In: Molecular Biology of the mycobacteria, Edited by McFadden, J. Surrey University Press. pp1-35.

Young, D.B., and S.T. Cole. 1993. Leprosy, tuberculosis, and the new genetics. J. Bacteriol. 175:1-6.

Young, R.A., V. Mehra, D. Sweetser, T. Buchanan, J. Clark-Curtiss, R.W. Davis, and B.R. Bloom. 1985. Genes for the major protein antigens of leprosy parasite *Mycobacterium leprae*. Nature (London) 316: 450-452.

Yphantis, D.A., and T. Arakawa. 1987. Sedimentation equilibrium measurements of recombinant DNA derived human interferon gamma. Biochemistry 26: 5422-27.

Chapter II

Secretion of Human Interleukin 2 by Recombinant *Mycobacterium bovis* BCG

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1. INTRODUCTION

It has been estimated that 1 billion people in the world have been infected by *M. tuberculosis* and approximately 3 million die of tuberculosis (TB) annually, especially in developing countries (Styblo, 1989). Bacille Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, is the vaccine used for prevention of tuberculosis. Even though more than 2 million people are vaccinated with BCG annually, the efficacy is highly variable from 0 to 80% (Fine, 1989). With the emergence of multiple-drug-resistant strains of mycobacteria, it is imperative to develop an improved vaccine for the prevention of mycobacterial infections. Despite the variable efficacy, BCG is an attractive vaccine vehicle due to a long history of safe use, an ability to act as an adjuvant and the development of shuttle vectors to perform molecular manipulations (Aldovini and Young, 1990). BCG has been used in expressing many foreign genes ranging from the gag, env and pol genes of HIV-1 (Aldovini and Young, 1991; Winter *et al.*, 1991) to a β-galactosidase gene from *E. coli* (Stover *et al.*, 1991). In addition, a B cell epitope of HIV-1 gag gene and an outer surface lipoprotein A (OspA) gene of *Borrelia burgdorferi* have been expressed and secreted from BCG as fusion proteins with the alpha(α)-antigen of BCG (Matsuo *et al.*, 1990; Stover *et al.*, 1993).

Although interleukin 2 (IL-2) was originally described as a T cell growth factor (Morgan *et al.*, 1976), it was later found to play a pivotal role in the immune system and to have pleiotropic effects on

different immune cells (Smith, 1988). IL-2 has been used in the treatment of various cancers (Rosenberg, 1988) and has been suggested for the treatment of mycobacterial infections (Jeevan and Asherson, 1988). It has been shown that administration of IL-2 *in vivo* can limit the replication of *Mycobacterium lepraemurium* (Jeevan and Asherson, 1988) and *Mycobacterium avium* in mice (Bermudez *et al.*, 1989). The toxicity associated with the use of high dose IL-2 and its very short half-life *in vivo* limit its use (Rosenberg, 1988). The expression and secretion of biologically functional IL-2 from recombinant BCG may overcome these problems. The secreted IL-2 may stimulate both humoral and cellular immune responses against mycobacterial or other recombinant proteins coexpressed in BCG to improve vaccine efficacy.

Recently, mouse and rat IL-2 proteins have been expressed and secreted from recombinant BCG strains by using the plasmid expression vector pMV261 (O'Donnell *et al.*, 1994). It was found that the IL-2 expressing-BCG can enhance the mouse interferon gamma (mIFN-γ) production by splenocytes *in vitro*. Whether this result can be translated into *in vivo* efficacy remains to be seen.

In this Chapter, I report the expression and secretion of biologically active huIL-2 from recombinant BCG along with *in vivo* data supporting the use of cytokine-secreting BCG to augment host immune responses against mycobacteria.

2. MATERIALS AND METHODS

Bacterial strains, plasmids

BCG Pasteur strain and *E. coli*/BCG shuttle plasmid vectors pMV261, pMV306 and pAB26 were kindly provided by Scott Koenig (MedImmune, Inc., Maryland, USA (Stover *et al.*, 1991). The 4 kb plasmid pMV306 is a derivative of pMV361 (Stover *et al.*, 1991), in which the expression cassette was replaced by a multiple cloning site (MCS). BCG cells were grown in Dubos media (Difco Laboratories, Detroit, MI) supplemented with 10% ADC (5% BSA fraction V, 2% dextrose and 0.85% NaCl).

Oligonucleotide primers.

Oligonucleotide primers were purchased from Dept. of Biological Sciences, University of Alberta, who synthesized them by using a DNA synthesizer (Applied Biosystems, Inc., Foster city, Calif.). Different primers used in constructing the huIL-2 expression plasmids are shown in the Table 2.1.

Construction of expression plasmids.

The plasmids used in expressing huIL-2 are shown in Fig. 2.1; the vectors, pMV261 and pMV306, are shown in panel A while three expression cassettes, CI (huIL-2), CII (huIL-2) and CIII (huIL-2), are shown in panel B. Insertion of the three expression cassettes into the multiple cloning sites (MCS) of vector pMV261 and pMV306 generates six different plasmids. The CI (huIL-2) was inserted into the KpnI and HindIII sites of the vectors while CII (huIL-2) and CIII

(huIL-2) were inserted into the XbaI and HindIII sites of the vectors.

Expression cassette CI (huIL-2) was constructed as follows: a DNA fragment containing the α-antigen promoter and signal peptide was synthesized by PCR with primers DKU56 and DKU57 from Pasteur strain BCG genomic DNA. An EcoRI site was added to its 5' end and a 10bp sequence identical to the huIL-2 5' encoding region was added at the 3' end. The huIL-2 encoding region was also synthesized by PCR with primers DKU57 and DKU59 from a pCD-huIL-2 (Taniguchi *et al.*, 1983) plasmid by adding a HindIII site at its 3' end and a 10 bp sequence identical to the α-antigen signal peptide 3' region at its 5' end. These two DNA fragments were spliced together by overlap extension by PCR using primers DKU56 and DKU59 as described (Horton *et al.*, 1989), and then digested with EcoRI and HindIII and cloned into pBluescriptSK(+) (Stratagene, Cloning Systems, LA Jolla, Calif. USA). After the sequence was confirmed by DNA sequencing (Sanger *et al.*, 1977), the DNA fragment containing the α-antigen-huIL-2 sequence was cloned into the KpnI and HindIII sites of the pMV306 and pMV261 vectors.

Expression cassette CII (huIL-2) was constructed from CI and another plasmid pAB26 (Stover *et al.*, 1993) which has the α-antigen gene linked to the heat shock protein 60 (hsp60) promoter of BCG. Briefly, an XbaI-SacII fragment having the hsp60 promoter and part of the α-antigen signal peptide sequence was obtained from pAB26 and ligated to a SacII-HindIII fragment having the whole IL-2 encoding sequence and part of the α-antigen signal peptide sequence

from CI, and cloned into the XbaI-HindIII sites of pMV306 and pMV261.

The expression cassette CIII was constructed by ligating the PCR-synthesized huIL-2 encoding sequence to the BamHI and HindIII sites located downstream of the hsp60 regulatory sequences of pMV261. Briefly, the huIL-2 encoding sequence was synthesized with PCR using primers DKU59 and DKU64 from a pCD-huIL-2 plasmid (Taniguchi *et al.*, 1983), and then cloned into the BamHI and HindIII sites of pBluescriptSK(+) for sequencing. After the sequences were confirmed by DNA sequencing (Sanger *et al.*, 1977), the huIL-2 encoding sequence was cloned into pMV261 and then the whole expression cassette was cut out with XbaI and HindIII and cloned into pMV306.

Electroporation

Various expression plasmids were transformed into the BCG Pasteur strain by electroporation as described (Stover *et al.*, 1993). To make competent BCG cells for electroporation, 250 ml of BCG cells, grown in Dubos medium supplemented with 10% ADC to log growth phase, were centrifuged for 10 min. at 1,000g. The pellet was washed twice with 10% glycerol, and then suspended in 2.5 ml of 10% glycerol. The competent BCG cells were aliquotted into 50 µl and frozen at -70°C. For electroporation, 1 µg plasmid DNA was mixed with 50 µg of BCG competent cells and placed on ice for 1 min. The DNA and BCG mixture was put into a 0.2 cm electrode gap cuvette (Bio-Rad) and electroporated using the pulser (Bio-Rad, Richmond,

CA) at 2500V, 25 mF and the pulse controller at 1000 ohms. The transformed BCG cells were plated on Middlebrook-7H10 agar supplemented with 10% ADC (5% BSA fraction V, 2% dextrose and 0.85% NaCl) and 15 µg/ml Kanamycin. After growth for 2-3 weeks at 37°C, individual colonies were picked and grown in Dubos media supplemented with 10% ADC.

BCG genomic DNA purification

Genomic DNA was isolated from BCG and various recombinant BCG strains by a glass bead homogenization method as described (Jacobs *et al.*, 1991). 50 ml of BCG cells, grown in Dubos with 10% ADC to log growth phase, were centrifuged in a 50 ml polypropylene tube at 1,000g for 10 min. The pellet was suspended in 1 ml homogenizing buffer (0.3 M Tris, pH 8.0, 0.1 M NaCl and 6 mM EDTA), and transferred to a 15 ml polypropylene tube containing 0.5-1 ml of pre-siliconized 40 mesh glass beads (BDH, Toronto, ON.). The mixture of BCG cells and glass beads was vortexed vigorously for about 6-10 min., then extracted twice with 2 ml phenol, once with 1:1 phenol/chloroform and once with chloroform. The DNA was precipitated with 2.5 volumes of 95% ethanol. After drying under vacuum, 100 µl TE and 1 µl RNase (10 mg/ml) was added.

Southern transfer

Approximately 1 µg genomic DNA isolated from BCG and various recombinant BCG strains was digested with 10-20 units of restriction enzyme(s) in a 30 µl reaction mixture using buffer

supplied by the manufacturer. The digested genomic DNA was separated in a 1% agarose gel. Appropriate amounts of agarose were dissolved in TAE solution (40 mM Tris-acetate, 1 mM EDTA) in a microwave oven. After cooling to 65°C, ethidium bromide was added to a final concentration of 0.5-1 μg/ml before the gel was poured. The DNA samples were loaded into gel wells with the gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol for a 6x buffer). The HindIII digested lambda DNA fragments (23.13 kb, 9.42 kb, 6.68 kb, 4.36 kb, 2.32 kb, 2.03 kb, 0.564 kb and 0.125 kb) was used as a molecular weight standard.

Genomic DNA separated in an agarose gel was transferred to Hybond-N nylon membrane (Amersham, Oakville, ON.) according to the supplier's instructions. After a photograph was taken under ultraviolet illumination, the gel was immersed in a solution (0.4 N NaOH and 0.6 M NaCl) for about 30 minutes at room temperature with gentle agitation to denature the DNA. Then the gel was neutralized in a solution of 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5) for 30 minutes. The DNA was transferred to a Hybond-N membrane by capillary diffusion for 16-24 hours (Southern, 1975). After the transfer, the membrane was carefully removed away from the gel, and then subjected to Ultraviolet (UV) cross linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA.), and then dried at room temperature.

Mini-preparation of plasmid DNA

Mini-preparation of plasmid DNA was carried out according to the procedures described in Sambrook *et al.* (1989). About 1.5 ml of bacterial cells, grown overnight in Luria-Bertani (LB) medium containing 100 μg/μl ampicillin, was transferred to a 1.5 ml microfuge tube and centrifuged 30 seconds at 12,000g. The supernatant was discarded and the pellet was resuspended in 90 μl lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0). Then 10 μl of 20 mg/ml freshly prepared lysozyme (dissolved in lysis buffer) was added to the cell suspension, and then placed on ice. After 30 minutes, 200 μl alkaline SDS solution (0.2 M NaOH, 1% SDS) was added and incubated on ice for 5 minutes. 150 μl of 3 M KAc was added, and put on ice for 10 minutes and then centrifuged for 5 minutes at 12,000g. The supernatant was transferred to a new microfuge tube and extracted once with an equal volume of phenol, then phenol-chloroform (1:1) and chloroform. The DNA was precipitated by adding 1 ml 95% ethanol, washed with 70% ethanol and dried for about 30 minutes. The DNA was resuspended in 50 μl of TE buffer.

Isolation of DNA fragments from LMP gel

About 2 μg plasmid pMV306-huIL-2 was digested with BamHI and HindIII in a 20 μl reaction mixture, and then separated on a 1% low melting point (LMP) agarose gel. When sufficient separation was achieved, the 0.4 Kb huIL-2 DNA fragment was cut out of the gel with a razor blade and then stored in an Eppendorf tube. An

appropriate amount (1.5 ml/gm gel) of double distilled water was added to the gel piece. The tube was boiled for 10 minutes, and then kept at -20°C. The isolated huIL-2 DNA fragments were used to probe the Southern blot of the genomic DNA isolated from various recombinant BCG strains.

DNA labeling

The probes for DNA hybridization were labeled by using a Random Primed DNA Labeling Kit (Boehringer Mannheim). 50-200 ng pMV306 plasmid DNA or isolated huIL-2 DNA fragments were added to a 50 µl reaction mixture containing 1 µl dATP (0.5 mmol/l), 1 µl dGTP (0.5 mmol/l), 1 µl dTTP (0.5 mmol/l), 2 µl hexanucleotide mixture in 10 X concentrated reaction buffer, 5 µl [α^{32}P] dCTP (3000 Ci/mmol), and 1 µl Klenow enzyme and incubated at 37°C for 2 hours to overnight. The reaction was stopped by adding 2 µl EDTA, 0.2 mM (pH 8.0). The unincorporated nucleotides were removed by passing through a Sephadex G-50 column, as described in Sambrook *et al.* (1989).

DNA hybridization

The membrane (15 x 20 cm) was prehybridized at 42°C for at least 2 hours in a rolling bottle with 10 ml of hybridization solution containing 5 x SSC (3 M sodium chloride, 0.3 M sodium citrate), 0.5% SDS, 10% dextran, 5mM EDTA, 10X Denhardt's solution, 50 µg/ml denatured salmon sperm DNA and 50% formamide. After the prehybridization, the appropriate amount of α^{32}P-labelled probe was

added. The hybridization reaction continued for 12-24 hours at 42°C with constant rolling.

After hybridization, the membrane was washed twice with 2 X SSC, 0.1% SDS at room temperature, twice with 2 X SSC, 0.1% SDS at 65°C for 30 minutes each, twice with 0.1 X SSC, 0.1% SDS at 65°C for 30 minutes each, and then the damp membrane was wrapped with Saran wrap and subjected to autoradiography.

For hybridization of the immobilized genomic DNA to another probe, the Hybond-N membrane was boiled in distilled water for 30 minutes with gentle agitation and then dried at room temperature. The new hybridization was carried out as described above.

Human IL-2 bioassay.

2 X 10^8 BCG-CII cells were inoculated into 200 ml of Dubos medium supplemented with 10% ADC and grown in a 1000 ml roller bottle at 37°C for 21 days. On day 3 and every 2 or 3 days subsequently, 10 ml of this culture was harvested. The bacterial cells were centrifuged at 12,000g and the wet weight of the bacterial pellet was determined. The supernatant was filtered through a 0.45 µm membrane.

The huIL-2 activity from the supernatant was measured by using an IL-2 dependent HT-2 cell line with a colorimetric method as described (Mosmann, 1983). The HT2 cells, grown in the presence of huIL-2 (50 units/ ml), were harvested and then washed twice by centrifugation at 100g for 5 min. in RPMI 1640 and resuspended at 5 X 10^5 cells/ml in RPMI 1640 containing 10% FBS. Different samples

and standard preparations were diluted in a 96-well microtitration plate (Nunc, Denmark). 100 µl of resuspended HT2 cells were added to each well to give a cell number of 5 X 10^4 cells/well. The plate was incubated at 37°C in a 5% CO_2 atmosphere for 18-24 hours, and then pulsed for 4 hours with 10% of the tetrazolium dye MTT (Sigma, St. Louis). The resulting blue to dark product was dissolved in 0.004 N HCl made in isopropanol and the color was read at 540 nm using a Titertek Multiskan MC plate reader (Helsinki, Finland). The IL-2 activity was converted into standard units as described (Gearing *et al.*, 1987). To confirm that the activity in the supernatant of the BCG-CII culture was due to huIL-2, 0.5 µg goat anti-huIL-2 polyclonal neutralizing antibody was used in a 200 µl HT-2 cell IL-2 assay.

Infection of mice.

BALB/cCrAlt female mice (5-6 weeks old), obtained from the University of Alberta colony and given sterile food and acidified water, were inoculated intravenously through the tail vein with approximately 10^5 BCG, BCG-CI, BCG-CII and BCG-pMV306 suspended in 0.1 ml PBS. At predetermined intervals, groups of mice (4-5 mice/group) were sacrificed. The spleens from these mice were removed, weighed, and then homogenized in 5 ml of PBS. The spleen homogenates were diluted in PBS and then plated on 7H10 agar plates. After 2-3 weeks, the number of BCG colonies were counted. Differences among experimental groups were evaluated by Student's t test.

3. RESULTS

Construction of recombinant BCG strains expressing huIL-2.

To express and secrete huIL-2 from BCG, six different plasmids pMV261-CI (huIL-2), pMV261-CII (huIL-2), pMV261-CIII (huIL-2), pMV306-CI (huIL-2), pMV306-CII (huIL-2) and pMV306-CIII (huIL-2) were made as described in Materials and Methods. In pMV261-CI (huIL-2) and pMV306-CI (huIL-2), the huIL-2 encoding region was under the control of the α-antigen promoter of BCG, while the expression of the huIL-2 gene was regulated by the hsp60 promoter in pMV261-CII (huIL-2) and pMV306-CII (huIL-2). A signal peptide sequence isolated from the naturally secreted α-antigen of BCG (Matsuo *et al.*, 1988) was inserted between the promoter and the huIL-2 encoding region to facilitate secretion. In these four plasmids, the huIL-2 encoding sequence was fused directly to the α-antigen signal peptide sequence by gene splicing by overlap extension (Horton *et al.*, 1989). In pMV261-CIII (huIL-2) and pMV306-CIII (huIL-2), the huIL-2 encoding sequence was inserted into the BamHI and HindIII sites of the pMV261 expression cassette. In these plasmids, fusion proteins with 8 amino acids derived from the hsp60 protein of BCG and the BamHI site would be expressed. In plasmids pMV261-CI, CII and CIII, the expression cassettes were cloned into the *E. coli*/BCG shuttle plasmid vector pMV261. The plasmid pMV261 contains an *E. coli* origin of replication (oriE), a mycobacterial origin of replication (oriM), a Kanamycin resistant gene (aph) and an expression cassette, which contains 404 bp of the 5' regulatory sequences of BCG hsp60 gene

including the first six amino acids of the hsp60 protein of BCG. In plasmids pMV306-CI, CII and CIII, the expression cassettes were inserted into the integrative vector pMV306. pMV306 contains an attachment (attP) site and an integrase (int) gene derived from mycobacteriophage L5, which allows the integration of the vector into the BCG genome by site-specific recombination (Stover *et al.*, 1991).

All six expression plasmids were transformed into the BCG Pasteur strain by electroporation as described in the Materials and Methods. As shown in the Table 2.2, no colonies could be obtained form the electroporation of all three plasmids using the vector pMV261 while approximately 500-1000 colonies could be obtained when the plasmids using integrative vector pMV306 were transformed into BCG, which is similar to the number of colonies obtained from the vector controls (pMV306 and pMV261).

The integration of the huIL-2 gene and the vector pMV306 into the BCG genome generated 4 different BCG strains, BCG-CI (huIL-2), BCG-CII (huIL-2), BCG-CIII (huIL-2) and BCG-pMV306, in which the plasmids pMV306-CI, CII, CIII and pMV306 respectively were integrated into the BCG genome. Southern blot and hybridization results (Figure 2.2) using the huIL-2 gene and pMV306 as probes are consistent with the restriction map shown in Figure 2.1 and indicate that the plasmids pMV306-CI, CII and pMV306 were integrated into the same region of the BCG genome as expected. A KpnI site was located 0.3 Kb to the left of the inserted DNA fragments and another KpnI site was located approximately 2 Kb to the right.

Expression and secretion of huIL-2 from recombinant BCG.

In the supernatant of a BCG-CII (huIL-2) culture, approximately 150 units of active huIL-2 was detected as presented in Table 2.2 and in Figure 2.3a. The huIL-2 activity could be neutralized by goat anti-huIL-2 polyclonal antibody (Figure 2.3b). An important distinction is whether the huIL-2 in the supernatant of the BCG-CII cultures is due to the release of huIL-2 from the autolysis of BCG cells or due to secretion. It has been shown that the autolysis of BCG cells generally occurs during the late log growth phase (Wiker *et al.*, 1991), and that the proteins released from the autolysis of BCG cells normally appear at this stage, and then increased rapidly. To study the secretion of huIL-2 from recombinant BCG-CII (huIL-2), I followed the growth of BCG-CII (huIL-2) and the IL-2 activity in the supernatant of the liquid culture at various days. As shown in Figure 2.3a, approximately 6 units/ml of huIL-2 was detected at 3 days prior to the log growth phase and then the IL-2 level rapidly increased during the log growth phase. The huIL-2 activity in the supernatant corresponded closely with the BCG-CII growth (Figure 2.3a) and reached a plateau of approximately 150 units/ml. The BCG-CI, with the α-antigen promoter instead of the hsp60 promoter used in BCG-CII, expressed approximately 25 units of huIL-2, that is 1/6 the amount of huIL-2 compared to BCG-CII.

Growth of huIL-2 expressing BCG in mice.

To test whether the huIL-2-expressing BCG have any effects on the growth of BCG *in vivo*, we infected groups of BALB/cCrAlt mice intravenously with 1.1 x 10^5 colony forming units (cfu) of various BCG strains and monitored the BCG growth in these mice. There was no difference in the seeding of the bacteria as the number of BCG cfu in the spleens of mice injected with BCG-CII and BCG-pMV306 control at 24 hours after infection was the same (Figure 2.4c). At week 3, the viable bacterial counts in the spleens of mice infected with BCG-CII were significantly less than the counts in the spleens of mice infected with BCG ($p < 0.005$) or BCG-pMV306 ($p < 0.001$). The growth of the mycobacteria in the spleens correlated with splenic enlargement (Figure 2.4b) and are consistent with a previous report (Sher *et al.*, 1973). Starting from week 1 to the end of the experiment (week 6), the growth of bacteria was significantly less in the spleens of mice infected with BCG-CII compared to the control BCG-pMV306 (at week 1, $p = 0.013$)(Figure 2.4c).

Growth of huIL-2 expressing BCG *in vitro*

As described in the last section, the BCG-CI, BCG-CII and BCG-pMV306 have a different growth rate in BALB/c mice, To test whether these three rBCG strains have a different growth rate *in vitro*, their growth was monitored in Dubos medium supplemented with 10% ADC. The results are shown in the Figure 2.5. The growth rate for these three rBCG strains is quite similar, with a doubling time of approximately 2 days.

4. DISCUSSION

Initially, an autonomously replicating plasmid vector pMV261 (Stover *et al.*, 1991) was used unsuccessfully in an attempt to express huIL-2. When the plasmids pMV261 having either the huIL-2 gene or the huIL-2 gene with the α-antigen signal peptide under the control of hsp60 promoter (Table 2.2) were electroporated into BCG, no colonies grew on the 7H10 Kanamycin selection plates, while 1 μg of pMV261 control DNA generally gave rise to 500-1000 colonies. Thus the huIL-2 gene could not be expressed in BCG Pasteur strain with the shuttle plasmid vector pMV261. On the other hand, the huIL-2 gene with or without the α-antigen signal sequence can be maintained in the genome of BCG by using the integrative vector pMV306 (Table 2.2). This provides an example of being able to maintain a foreign gene, which was not easily established with plasmid vectors, by using an integrative vector in BCG just as described with other bacterial systems (Saunders *et al.*, 1984). A likely explanation for this phenomenon is over-expression lethality (Burlein *et al.*, 1994).

As shown in Table 2.2, no huIL-2 activity was detected from either supernatant or cell sonicates of BCG-pMV306-CIII. It is possible that the α-antigen-huIL-2 fusion protein expressed by BCG-CIII is not functional. Structure-function analysis of huIL-2 by Ju *et al.*, (1987) identified three regions, which are important for the biological activities of huIL-2, one of which is the 1-20 amino acids of the NH_2 terminus. Therefore, it is possible that the addition of 8

amino acids to the N-terminus of huIL-2 protein blocks its function. To confirm whether a nonfunctional fusion IL-2 protein was expressed, more experiments, such as ELISA and Western blotting, could be done.

Mouse IL-2 and rat IL-2 have recently been expressed using the plasmid vector pMV261 (O'Donnell *et al.*, 1994). It was also found that the BCG transformation efficiencies for pMV261 having the mouse and rat IL-2 genes without signal sequences were 10 to 100-fold less than the IL-2 constructs having the α-antigen signal sequence. The difference in the ability to express the murine and human IL-2 genes with pMV261 in BCG may be due to the IL-2 sequence diversity between these species (Yokota *et al.*, 1985). The predictors of success for expressing proteins in BCG with these expression vectors are currently unknown although expression seems to be somewhat gene and protein specific (personal communication, Scott Koenig, MedImmune, Inc., Maryland, USA). With the use of the integrative vector pMV306, the huIL-2 gene in both plasmids CI and CII was integrated into the BCG genome. The southern hybridization results show that CI, CII and pMV306 vector control were integrated into the same chromosomal region of BCG. These results are consistent with previous studies (Lee *et al.*, 1991), which showed that the phage L5-based vectors integrate site-specifically into a tRNA region of the mycobacterial genome. This integration does not appear to disrupt any essential function of any gene (Lee *et al.*, 1991).

In the supernatant of a BCG-CII culture, approximately 150 units/ml huIL-2 can be detected by using the HT-2 bioassay. More than 95% of huIL-2 activity was present in the medium while less than 5% of the huIL-2 activity was detected from the BCG-CII cell sonicates (Table 2.2), and it has been shown that the extracts of BCG did not contain any IL-2 inhibitor (O'Donnell *et al.*, 1994). The huIL-2 activity can be detected prior to log phase growth and corresponded closely with the BCG-CII growth (Figure 2.3a). Consistent with previous studies on the secretion of proteins from BCG and *M. tuberculosis* (Wiker *et al.*, 1991), the huIL-2 detected from the BCG-expressing strain culture is due to the secretion of huIL-2 from BCG cells and not due to the autolysis of BCG cells.

BCG-CI, in which the huIL-2 gene was under the control of BCG α-antigen promoter instead of the BCG hsp60 used in BCG-CII, expressed approximately 1/6 the amount of huIL-2 expressed by BCG-CII. This indicates that the BCG hsp60 promoter is a better promoter than the BCG α-antigen promoter in driving expression and secretion of huIL-2. Consistent with this observation, we have found that hsp60 is also a stronger promoter than the BCG α-antigen promoter in directing the expression of other cytokines (Chapter III and IV).

The heat shock protein promoters are the most commonly used promoters in expressing foreign genes in mycobacteria (Burlein *et al.*, 1994). The expression of hsp60 protein is constitutive under all growth conditions, and is highly induced with moderate heat shock (37°C) (Young *et al.*, 1994). With the use of the hsp60 promoter,

foreign genes can be expressed to 10% or more of the total mycobacterial protein in BCG (Stover *et al.*, 1991). Even though the α-antigen promoter directs the expression and secretion of large amounts of α-antigen proteins during the early phases of growth of mycobacteria (Young *et al.*, 1994), we found that the α-antigen promoter serves as a poor promoter in directing the expression of huIL-2 in BCG Pasteur strain. The reasons for this discrepancy are unknown. Since the promoter of the α-antigen was only deduced from sequence comparison (Matsuo *et al.*, 1988), the exact DNA sequences involved in the regulation of α-antigen expression are unknown. As only 90 bp of the 5' regulatory sequences of the α-antigen are included in our promoter, it is possible that some regulatory sequences required for high expression of the α-antigen are missing from the promoter that we used.

It has been shown that IL-2 plays an important role in mycobacterial infections and that the administration of IL-2 in mice can make mice more resistant to mycobacterial infections (Bermudez *et al.*, 1989; Jeevan *et al.*, 1988). Therefore we studied the growth of the huIL-2-expressing BCG in the spleens of mice to test whether the huIL-2-expressing-BCG can limit the growth of BCG in *vivo*. We found that starting from week 1 to the end of the experiments, the bacterial numbers in the spleens of mice infected with BCG-CII were significantly less than those in the spleens of mice infected with various control BCG strains (Figure 2.4). This difference is not likely due to a different growth rate of the different BCG strains as there was no difference in the growth rates of the different BCG strains *in*

vitro (Figure 2.5). If the actual insertion of pMV306-huIL-2 affected the *in vivo* growth rate, the same effect would be expected with control BCG-pMV306, in which the vector pMV306 was inserted into the same region as BCG-CII. There was no significant difference in the bacterial counts of mice infected with BCG and BCG-pMV306. Moreover, when the same number of BCG-CI, which expresses approximately 1/6 the amount of huIL-2 as BCG-CII, was injected into the mice, the number of bacterial cfu recovered from the spleens of these infected mice was significantly less than the number of cfu recovered from mice infected with BCG (P=0.02, Figure 2.4a) or BCG-pMV306 (P=0.01, Figure 2.4a), but was significantly greater than those with BCG-CII (P=0.007 Figure 2.4a). The lower number of cfu of the huIL-2-expressing BCG strains recovered from the spleens of infected mice is likely due to the enhancement of the host immune response by the secreted huIL-2. The immune mechanisms involved in this process, particularly with respect to T cells and macrophages, are under investigation.

Even though I have shown that huIL-2-expressing rBCG lines have the same growth rate with the other control BCG lines *in vitro*, it is still possible that secretion of huIL-2 from rBCG can interfere with the secretion of other products of BCG, which may be necessary for BCG growth *in vivo*. To rule out this possibility, a rBCG line secreting nonfunctional huIL-2 could used as a control in future experiments.

I have found that the growth rates of different recombinant BCG lines in the spleens of infected BALB/c mice correlates with

splenic enlargement. But the cfu/per mg of spleen weight looks similar for mice infected with different BCG lines (Figure 2.4). Although the mechnism(s) are not clear, it has been shown that an increase in splenic weight was not due to the weight of the bacteria but rather due, in part, to cellular infiltration and cellular replication (Sher *et al.*, 1973). It is possible that the IL-2 secreted by rBCG results in a more effective immune response which would inhibit the growth and spread of BCG. The BCG controls might result in a less effective immune response, continue to proliferate, stimulating a further partially effective immune response, resulting in the splenic cellular infiltration and cellular replication, which has been shown to be responsible for the increase of spleen weight in the BCG-infected mice (Sher *et al.*, 1973).

M. tuberculosis is an intracellular pathogen that lives and multiplies in non-activated macrophages. Before *M. tuberculosis* can be destroyed by macrophages, these cells must be activated by T cells and/or lymphokines. IL-2 secreted from the IL-2-expressing BCG may activate macrophages directly (Maikovsky *et al.*, 1987; Strieter *et al.*, 1989) or indirectly by inducing the production of IFN-γ (Kawase *et al.*, 1983) and other cytokines (Belosevic *et al.*, 1990). It has been shown that recombinant BCG secreting mouse or rat IL-2 can increase the IFN-γ production of mouse spleen cells *in vitro* (O'Donnell *et al.*, 1994). The IL-2-expressing BCG may be a more effective vaccine for tuberculosis or may simply serve to help us to better understand the immune response to BCG.

BCG instillation is the treatment of choice for recurrences of superficial bladder cancer (Soloway and Perito, 1992). The combination of IL-2 and BCG in the therapy of superficial bladder cancer is more effective than either IL-2 or BCG alone in both a mouse model (Ikemoto *et al.*, 1991) and in a clinical trial (Cockett *et al.*, 1991). Therefore the IL-2-expressing BCG-CII may also have a role in the treatment of bladder cancer.

Recombinant cytokine-expressing BCG provides a model system for studying immunity to mycobacterial infections and in particular, the role of various cytokines. This system will provide information that will be invaluable in the development of immune modulators for the prevention and treatment of TB.

5. BIBLIOGRAPHY

Aldovini, A., and R.A. Young. 1990. Development of a BCG recombinant vehicle for candidate AIDS vaccines. Int. Rev. Immunol. 7: 79-83.

Aldovini, A., and R.A. Young. 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature 351: 479-482.

Belosevic, M., D.S. Finbloom, M.S. Meltzer, and C.A. Nacy. 1990. IL-2: A cofactor for induction of activated macrophage resistance to infection. J. Immunol. 145: 831-839.

Bermudez, L.E.M., P. Stevens, P. Kolonoski, M. Wu, and L.S. Young. 1989. Treatment of experimental disseminated *Mycobacterium*

avium complex infection in mice with recombinant IL-2 and tumor necrosis factor. J. Immunol. 143: 2996-3000.

Burlein, J.E., C.K. Stover, S. Offutt, and M.S. Hanson. 1994. Expression of Foreign Genes in Mycobacteria. In: Tuberculosis: Pathogenesis, Protection, and Immunity, edited by Bloom, B.R., ASM press, Washington, D.C. pp. 239-252.

Cockett, A.T.K., R.S. Davis, L.R. Cos, and L.L. Wheeless Jr. 1991. Bacillus Calmette-Guérin and interleukin 2 for treatment of superficial bladder cancer. J. Urol. 146: 766-770.

Fine, P.E. 1989. The BCG story: Lessons from the past and implications for the future. Reviews of Infectious Diseases 11, Sup. 2:353-359.

Gearing, A.J.H., and C.R. Bird. 1987. Production and assay of interleukin 2. In: lymphokines and Interferons: A practical approach, Oxford:IRL Press, pp291-301.

Horton, R.M., H.D. Hunt, S.N. Ho, J.K. Pullen, and L.R. Pease. 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61-68.

Ikemoto, S., S. Wada, T. Terada, M. Kamizuru, W. Sakamoto, T. Kishimoto, and M. Maekawa. 1991. Combined therapy of interleukin 2 with cyclophosphamide or Bacillus Calmette-Guérin against implanted bladder cancer cells in mice. Urol. Int. 47: 104-107.

Jacobs, W.R.J., G.V. Kalpana, J.D. Cirillo, L. Pascopella, S.B. Snapper, R.A. Udani, W. Jones, R.G. Barletta, and B.R. Bloom. 1991. Genetic systems for mycobacteria. Methods Enzymol. 204: 537-555.

Jeevan, A., and G.L. Asherson. 1988. Recombinant interleukin 2 limits the replication of *Mycobacterium lepraemurium* and *Mycobacterium bovis* BCG in mice. Infect. Immun. 56: 660-664.

Ju, G., L. Collins, K.L. Kaffka, W. Tsien, R. Chizzonite, R. Crowl, R. Bhatt, and P.L. Kilian 1987 Structure-function analysis of human interleukin 2. J. Biol. Chem. 262: 5723-5731.

Kawase, I., C.G. Brooks, and K. Kuribayashi. 1983. Interleukin-2 induces γ-interferon production: participation of macrophages and NK-like cells. J. Immunol. 131: 288-292.

Lee, M.H., L Pascopella, W.R. Jacobs Jr., and G.F. Hatfull. 1991. Site-specific integration of mycobacteriophage L5: integration-proficient vectors for *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and Bacille Calmette-Guérin. Proc. Natl. Acad. Sci. USA 88: 3111-3115.

Maikovsky, M., B. Loveland, M. North, G.L. Asherson, L. Gao, P. Ward, and W. Fiers. 1987. Recombinant interleukin 2 directly augments the cytotoxicity of human monocytes. Nature 325: 262-265.

Matsuo, K., R. Yamaguchi, A. Yamazaki, H. Tasaka, K. Terasaka, M. Totsuka, K. Kobayashi, H. Yukitake, and T. Yamada. 1990. Establishment of a foreign antigen secretion system in mycobacteria. Infect. Immun. 58: 4049-4054.

Matsuo, K., R. Yamaguchi, A. Yamazuki, H. Tasaka, and T. Yamada. 1988. Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular α antigen. J. Bacteriol. 170: 3847-3854.

Morgan, D.A., F.W. Ruscetti, and R. Gallo. 1976. Selective *in vivo* growth of T lymphocytes from normal bone marrows. Science 193: 1007-1008.

Mosmann, T.R. 1983. Rapid colorimetric assay for cellular growth and cytotoxicity assays. J. Immunol. Method 65: 55-63.

O'donnell, M.A., A. Aldovini, R.B. Duda, H. Yang, A. Szilvasi, R.A. Young, and W.C. Dewolf. 1994. Recombinant *Mycobacterium bovis* BCG secreting functional interleukin 2 enhances gamma interferon production by splenocytes. Infect. Immun. 62: 2508-2514.

Rosenberg, S.A. 1988. The development of new immunotherapies for the treatment of cancer using interleukin 2: A review. Ann. Surg. 208: 121-135.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467.

Saunders, C.W., B.J. Schmidt, M.S. mirot, L.D. Thompson, and M.S. Guyer. 1984. Use of chromosomal integration in the establishment

and expression of blaZ, a *Staphylococcus aureus* β-lactamase gene, in *Bacillus subtilis*. J. Bacteriol. 157: 718-726.

Sher, N.A., S.D. Chaparas, J. Pearson, and M. Chirigos. 1973. Virulence of six strains of *Mycobacterium bovis* (BCG) in mice. Infect. Immun. 8: 736-742.

Smith, K.A. 1988. Interleukin-2: inception, impact and implications. Science 240: 1169-1176.

Soloway, M.S., and P.E. Perito. 1992. Superficial bladder cancer: diagnosis, surveillance and treatment. J Cell Biochem. Suppl. 16I: 120-127.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Stover, C.K., G.P. Bansal, M.S. Hanson, J.E. Burlein, S.R. Palaszynski, J.F. Young, S. Koenig, D.B. Young, A. Sadzine, and A.G. Barbour. 1993. Protective immunity elicited by recombinant Bacille Calmette-Guérin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate lyme disease vaccine. J. Exp. Med. 178: 197-209.

Stover, C.K., V.F. De la Cruz, T.R. Fuerst, J.E. Burlein, L.A. Benson, L.T. Bennett, G.P. Bansal, J.F. Young, M.H. Lee, G.F. Hatfull, S.B. Snapper, R.G. Barletta, W.R. Jacobs, Jr., and B.R. Bloom. 1991. New use of BCG for recombinant vaccines. Nature 351: 456-460.

Strieter, R.M., D.G. Remick, J.P. Lynch, R.N. Spengler, and S.L. Kunkel. 1989. Interleukin-2-induced tumor necrosis factor-alpha (TNF-α) gene expression in human alveolar macrophages and blood monocytes. Am. Rev. Respir. Dis. 139: 335-342.

Styblo, K. 1989. Overview and epidemiologic assessment of the current global tuberculosis situation with an emphasis on control in developing countries. Rev. of Infect. Dis. 2: S339-S346.

Taniguchi, T., H. Matsui, T. Fujita, C. Takaoka, N. Kashima, R. Yoshimoto, and J. Hamuro. 1983. Structure and expression of a cloned cDNA for human interleukin 2. Nature 302: 305-310.

Wiker, H., M. Harboe, and S. Nagai. 1991. A localization index for distinction between extracellular and intracellular antigens of *Mycobacterium tuberculosis*. J.Gen.Microbiol. 137: 875-884.

Winter, N., M. Lagranderie, J. Rauzier, J. Timm, C. Leclerc, B. Guy, M.P. Kieny, M. Gheorghiu, and B. Gicquel. 1991. Expression of heterologous genes in *Mycobacterium bovis* BCG: induction of a cellular response against HIV-1 Nef protein. Gene 109: 47-54.

Yokota, T., N. Arai, F. Lee, D. Rennick, T. Mosmann, and K. Arai. 1985. Use of a cDNA expression vector for isolation of mouse interleukin 2 cDNA clones: Expression of T cell growth-factor activity after transfection of monkey cells. Proc. Natl. Acad. Sci. USA 82: 68-72.

Young, D., T. Garbe, R. Lathigra, and C. Abou-Zeid. 1994. Protein antigens: structure, function and regulation. In: Molecular Biology

of the Mycobacteria, edited by McFadden, J. London: Surrey University Press, pp1-35.

Table 2.1 Primers used for constructing the huIL-2 expression plasmids

DKU56: TATgaattcGCCCGAATCGACATTTG[1]
DKU57: agtaggtgcCGCGCCCGCGGTTGCCGCTC[2]
DKU58: ccgcgggcgcgGCACCTACTTCAAGTTCTAC[3]
DKU59: AGGaagcttAATTATCAAGTTAGTGTTG[4]
DKU64: CTCggatccGGCACCTACTTCAAGTTC[5]

Notes:

1. Lower case letters indicate the added EcoRI site.
2. Lower case letters indicate the sequence identical to the huIL-2 5' encoding sequence.
3. Lower case letters indicate sequence identical to α-antigen signal peptide 3' sequence.
4. Lower case letters indicate the added HindIII site.
5. Lower case letters indicate the added BamHI site.

Table 2.2 Expression of huIL-2 in BCG

	BCG Colonies (μg DNA)	Activity (Units)[a] in Supernatants	Activity (Units)[b] in Cell Sonicates
pMV261-CI(HuIL-2)	0		
pMV261-CII(HuIL-2)	0		
pMV261-CIII(HuIL-2)	0		
pMV306-CI(HuIL-2)	500-1000	25	ND[c]
pMV306-CII(HuIL-2)	500-1000	150 (95%)	< 8 (5%)
pMV306-CIII(HuIL-2)	500-1000	0	0

Notes:

a. Activity is from 1 ml saturated culture of various rBCG's. All samples were done in triplicate, and the stardard error of means were all less than 5%.

b. Activity is from the sonicates of 1 ml saturated culture of various rBCG and 1 ml saturated BCG cells generally weighs 10 mg.

c. ND means not done.

Figure 2.1 Plasmids used for expressing huIL-2 in BCG.

A. Two *E. coli*/BCG shuttle plasmid vectors pMV306 and pMV261 used for making expression plasmids. Explanation for these plasmid vectors can be found in the Results.

B. Three expression cassettes, CI (huIL-2), CII (huIL-2) and CIII (huIL-2), constructed for expressing huIL-2. Explanation for making these constructs can be found in the Materials and Methods of Chapter II.

Insertion of three expression cassettes into two vectors generates six expression plasmids, pMV261-CI (huIL-2), pMV261-CII (huIL-2), pMV261-CIII (huIL-2), pMV306-CI (huIL-2), pMV306-CII (huIL-2), pMV306-CIII (huIL-2). The expression cassette CI (huIL-2) was inserted into the KpnI and HindIII sites of the vectors; while the expression cassettes, CII (huIL-2) and CIII (huIL-2) were inserted into the XbaI and HindIII sites of the vectors.

The restriction enzymes shown in the map are: K, KpnI; P, PstI; E, EcoRI; H, HindIII; B, BamHI; Bg, BlgII; N, NotI; S, SalI; X, XbaI.

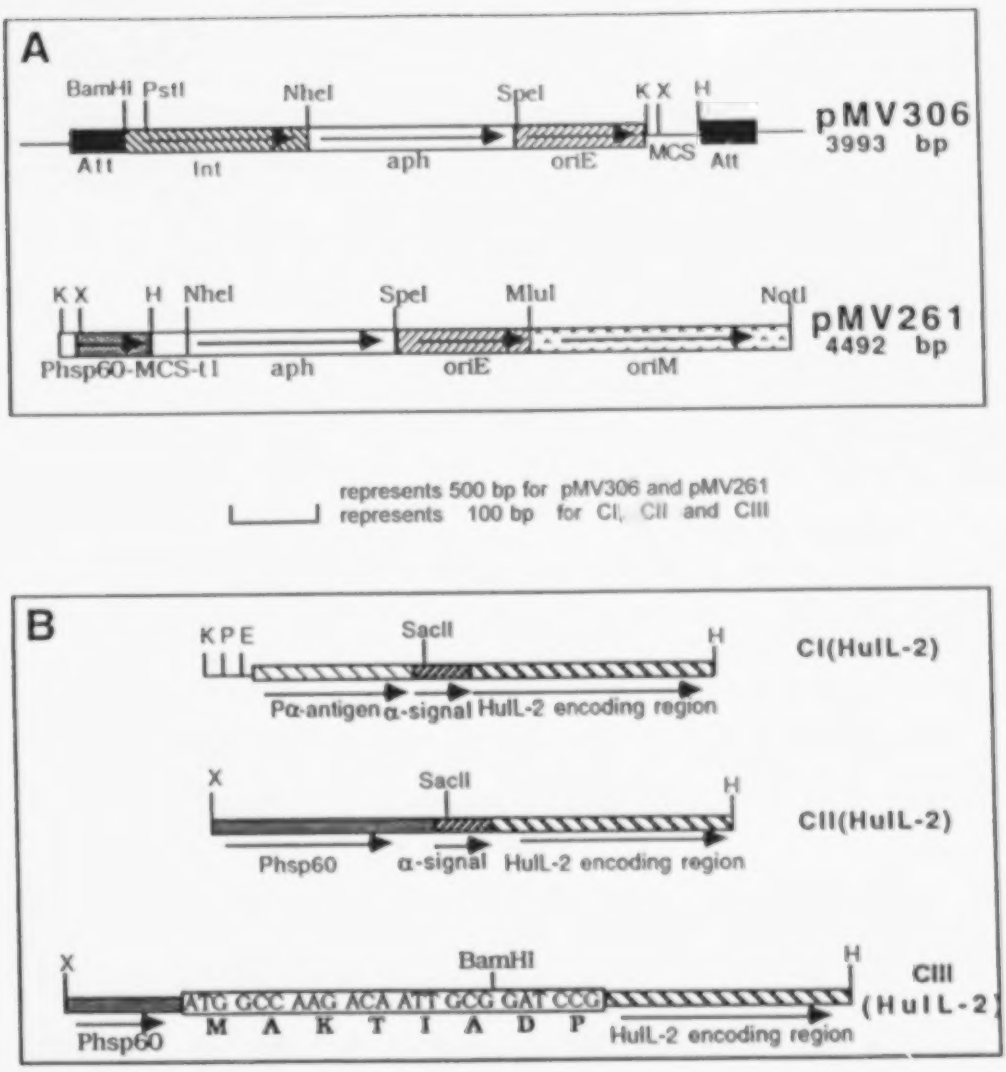

A
BamHI PstI
NheI
SpeI
K X H
pMV306
3993 bp
Att
Int
aph
oriE
MCS
Att
K X H NheI
SpeI
MluI
NotI
pMV261
4492 bp
Phsp60-MCS-t1
aph
oriE
oriM
represents 500 bp for pMV306 and pMV261
represents 100 bp for CI, CII and CIII
B
K P E
SacII
H
CI(HuIL-2)
Pα-antigen
α-signal
HuIL-2 encoding region
X
SacII
H
CII(HuIL-2)
Phsp60
α-signal
HuIL-2 encoding region
X
BamHI
H
CIII
(HuIL-2)
ATG GCC AAG ACA ATT GCG GAT CCG
M A K T I A D P
Phsp60
HuIL-2 encoding region

Figure 2.2. Southern blot and hybridization of genomic DNA from BCG-pMV306-CI (huIL-2) (lanes 1 and 2), BCG-pMV306-CII (huIL-2) (lanes 3 and 4), BCG-pMV306 (lanes 5 and 6) and BCG (lane 7), confirming the integration of all expression plasmids into the same region of the BCG genome.

The blot was first probed with pMV306 (left panel), and then with the huIL-2 encoding sequence (right panel). The genomic DNA in the lanes 1, 3, 5 and 7 was digested with KpnI while the DNA in lanes 2, 4 and 6 was digested with KpnI and HindIII.

The experiments were carried out as described in Materials and Methods.

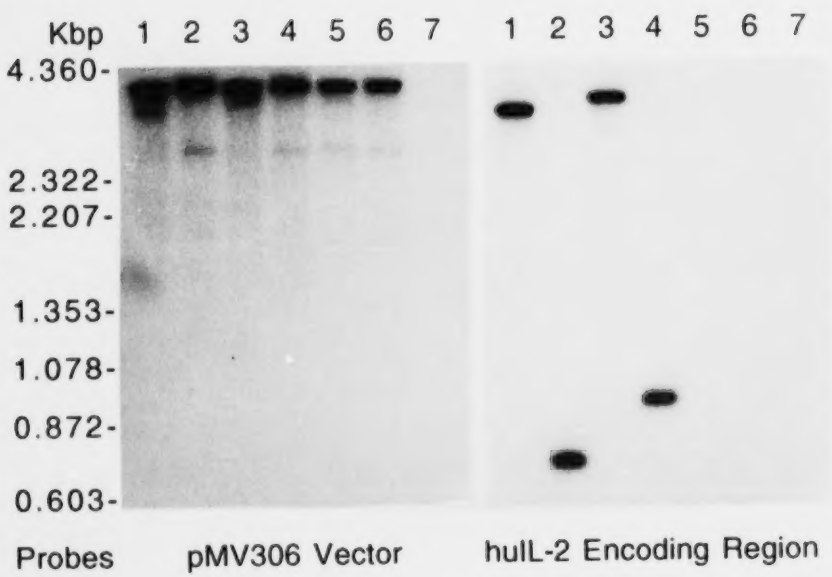

Kbp 1 2 3 4 5 6 7 1 2 3 4 5 6 7
4.360-
2.322-
2.207-
1.353-
1.078-
0.872-
0.603-
Probes
pMV306 Vector
hulL-2 Encoding Region

Figure 2.3 Expression and secretion of huIL-2 from BCG-pMV306-CII (huIL-2). The experiments were carried out as described in Materials and Methods. The results are given as means ± SEM (Standard Error of Mean) from three samples.

a. Growth of BCG-pMV306-CII (huIL-2) in Dubos medium and biological assay of IL-2 activity in BCG-pMV306-CII supernatant. No huIL-2 activity was detected from the BCG or BCG-pMV306 controls.

b. Neutralization of huIL-2 in BCG-pMV306-CII (huIL-2) supernatant harvested at day 21 with goat anti-huIL-2 polyclonal antibody.

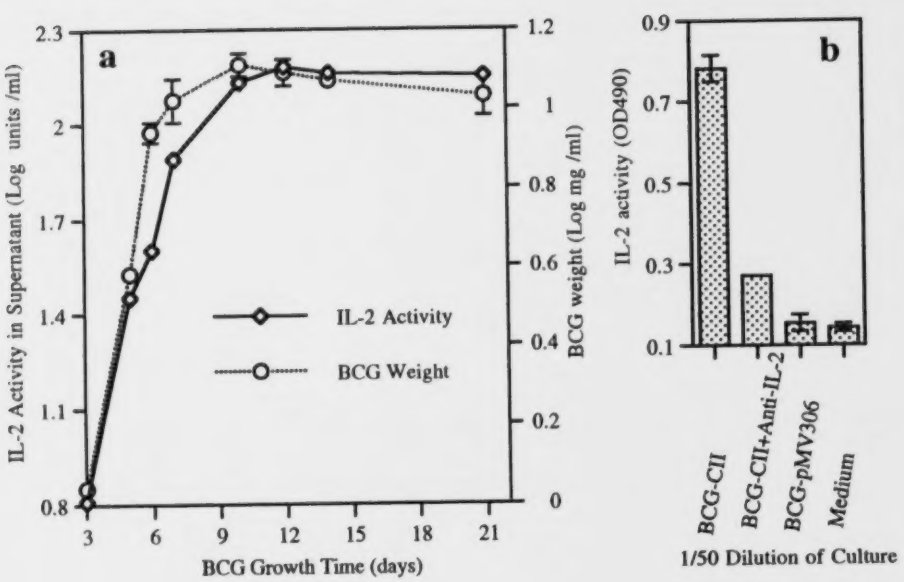

a
IL-2 Activity in Supernatant (Log units /ml)
BCG weight (Log mg /ml)
IL-2 Activity
BCG Weight
BCG Growth Time (days)
b
IL-2 activity (OD490)
BCG-CII
BCG-CII+Anti-IL-2
BCG-pMV306
Medium
1/50 Dilution of Culture

Figure 2.4 Infection of BALB/c mice with BCG Pasteur strain, BCG-pMV306-CI (huIL-2), BCG-pMV306-CII (huIL-2) and BCG-pMV306. The results are presented as means ± SEM for five mice at each time point.

a. Total viable bacteria counts in spleens at the third week after infection.

b. Spleen weights of BALB/c mice infected with various BCG strains at the third week.

c. Time course of BCG-pMV306-CII (huIL-2) and BCG-pMV306 infection in BALB/c mice.

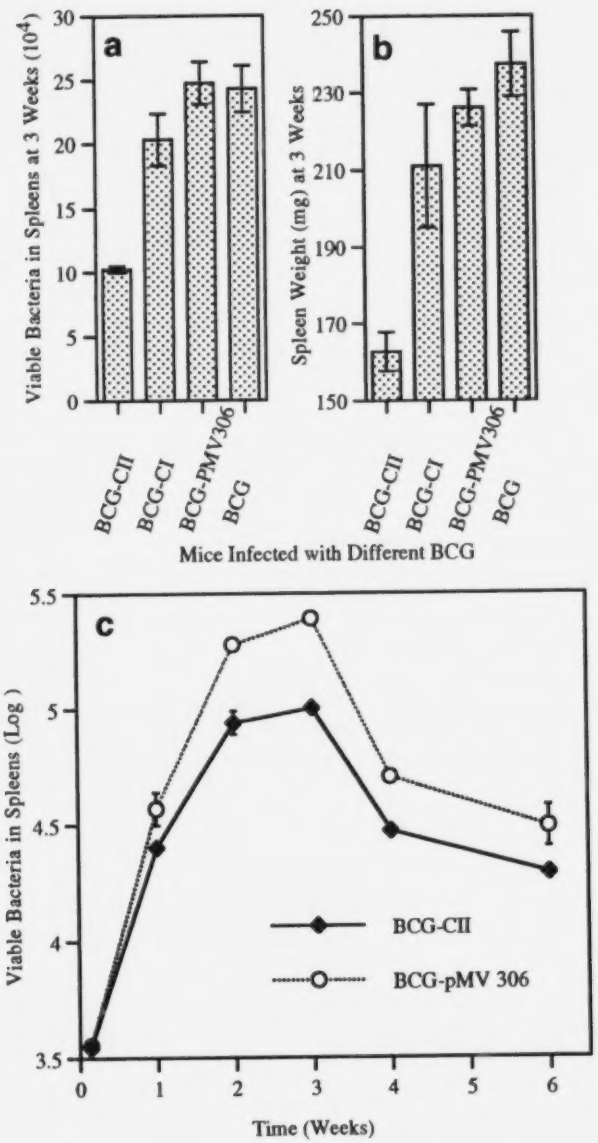
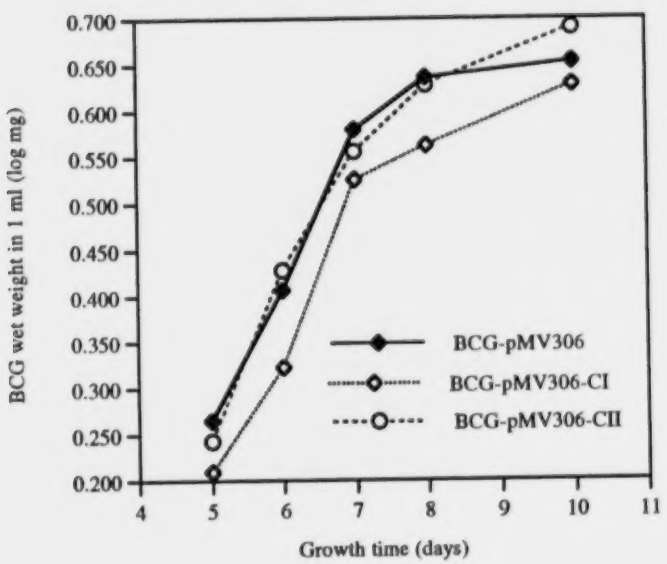

a
b
Viable Bacteria in Spleens at 3 Weeks (10^4)
Spleen Weight (mg) at 3 Weeks
30
25
20
15
10
5
0
250
230
210
190
170
150
BCG-CII
BCG-CI
BCG-PMV306
BCG
Mice Infected with Different BCG
c
Viable Bacteria in Spleens (Log)
5.5
5
4.5
4
3.5
0
1
2
3
4
5
6
Time (Weeks)
BCG-CII
BCG-pMV 306

Figure 2.5 *In vitro* growth of huIL-2-expressing BCG recombinants. 2 X 10^8 various BCG recombinants were inoculated into 200 ml of Dubos medium supplemented with 10% ADC (5% BSA fraction V, 2% dextose and 0.85% NaCl), and grown in a 1000 ml roller bottle at 37°C for 21 days. From day 3 and every 2 or 3 days subsequently, 10 ml of the cultures was harvested. The bacterial cells were spun down at 12,000g for 10 minutes, and the wet weight of BCG cells was determined. All samples were done in triplicate, and the standard error of means were all less than 5%.


0.700
0.650
0.600
0.550
0.500
0.450
0.400
0.350
0.300
0.250
0.200
4
5
6
7
8
9
10
11
BCG wet weight in 1 ml (log mg)
Growth time (days)
BCG-pMV306
BCG-pMV306-CI
BCG-pMV306-CII

Chapter III

HuTNF-α as a Reporter Gene to Study the Effects of Initiation Codon Change on Gene Expression in BCG

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1. INTRODUCTION

Mycobacterium bovis Bacillus Calmette-Guérin (BCG), attenuated by serial passage *in vitro* (Calmette *et al.*, 1924), is the most widely used vaccine for the prevention of tuberculosis (TB) in the world (Fine, 1989). A number of features of BCG, including its ability to act as a highly effective adjuvant and a long history of safe use make it an attractive vaccine vehicle (Aldovini and Young, 1990). Recently developed molecular genetic tools have allowed many foreign genes ranging from HIV-1 gag, pol and nef to *Escherichia coli* β-galactosidase to be expressed in BCG (Aldovini and Young, 1991; Murray *et al.*, 1992; Stover *et al.*, 1993 and Winter *et al.*, 1991). Moreover, foreign proteins delivered by recombinant BCG to mice can induce both cellular and humoral responses (Aldovini and Young, 1991; Murray *et al.*, 1992; Stover *et al.*, 1993). Molecular genetic studies on mycobacteria have lagged behind those in other bacteria partly because of the slow growth and the predisposition to clumping by mycobacterial cells. As a result, the regulation of gene expression in mycobacteria is largely unknown (Burlein *et al.*, 1994). Expression of foreign genes in a recombinant host is a complex process which can be influenced by a number of factors at both the transcriptional and posttranscriptional levels (Buell and Panayotatos, 1986). Identification of regulatory sequences involved in mycobacterial gene expression has been mainly based on the similarity of the DNA sequences with those of other bacteria (Dale and Patki, 1990). In order to develop BCG as a more effective and useful multivaccine vehicle, studies on gene expression and regulation in this organism

are needed. The most convenient method for studying gene expression and regulation involves the use of a reporter gene. When fused to various control elements, a reporter gene provides a way to assess the mechanisms involved in the regulation of gene expression (Slauch and Silhavy, 1991). The commonly used reporter gene β-galactosidase has been expressed in BCG (Murray *et al.*, 1992; Stover *et al.*, 1991), but a system to use it as a reporter gene in mycobacteria has not been published. The expression of the frequently used reporter gene alkaline phosphatase (Slauch and Silhavy, 1991) has not been described in mycobacteria.

In this chapter, I report the expression of human tumor necrosis factor alpha (huTNF-α) in BCG. The report will concentrate on the use of huTNF-α as a reporter gene to study the effects of translation initiation codon change on the efficiency of gene expression in BCG.

TNF-α, named for its ability to cause necrosis of tumor cells, is mainly produced by cells of the mononuclear phagocyte lineage (Fiers, 1991). HuTNF-α is a well studied cytokine and its gene has been cloned and expressed in various expression systems (Pennica *et al.*, 1984). By fusing huTNF-α encoding sequences to a heat shock 60 promoter of BCG in an *E. coli*/BCG shuttle plasmid vector pMV261, we found that biologically active huTNF-α can be expressed to a very high level (approximately 2.5 μg/10 mg wet weight of BCG cells). The bioassay of huTNF-α, based on its cytolytic effect on the tumor cell line L-929-8 (Branch *et al.*, 1991), is simple to perform, and is

extremely sensitive. This makes huTNF-α a very attractive reporter gene in studying gene expression and regulation in mycobacteria.

The translation initiation codon is usually AUG in *E. coli* and other well studied prokaryotes, although GUG and UUG are also used in some genes (Gold, 1988). GUG and UUG initiation codons in *E. coli* are used far less frequently than AUG, and it has been shown that the codons other than AUG provide less efficient initiation of translation in *E. coli* (Kozak, 1983). In some studies when an AUG initiation codon was mutated to a GUG or other initiation codon, the expression of these genes was markedly reduced (Looman and Knippenberg, 1986; Ringquist *et al.*, 1992). On the other hand, AUG and GUG initiation codons have been reported to be used almost equally in mycobacteria, probably reflecting the high G-C content (approximately 65%) of mycobacteria (Dale and Patki, 1990). The use of GUG as an initiation codon in mycobacteria was mainly deduced from the DNA sequences of cloned genes and has only been confirmed by comparison with the N-terminal amino acids in a few cases (Dale and Patki, 1990). The effect of initiation codon usage on correct initiation and the expression efficiency remains uncertain. To address these issues, the huTNF-α encoding sequence was fused to the transcriptional and translational regulatory sequences of the BCG hsp60 gene. When the AUG initiation codon in the hsp60 gene was changed to GUG by *in vitro* mutagenesis, we found that the expression of huTNF-α was increased at least 100 percent. This is the first example showing that GUG serves as a more efficient initiation codon than AUG.

2. MATERIALS AND METHODS

Bacterial strains, plasmids

Bacterial strains and plasmid vectors used during this study were described in the Materials and Methods of Chapter II.

Oligonucleotide primers.

Synthesis of Oligonucleotide primers were described in Chapter II. Different primers used in constructing the huTNF-α expression plasmids are shown Table 3.1.

Construction of expression plasmids.

The plasmids used in expressing huTNF-α are shown in Figure 3.1; the vectors, pMV261 and pMV306, are shown in panel A while three expression cassettes, CI (huTNF-α), CII (huTNF-α) and CIII (huTNF-α) are shown in panel B. Insertion of the three expression cassettes into the multiple cloning sites (MCS) of vector pMV261 and pMV306 generates six different plasmids. All expression cassettes were inserted into the XbaI and HindIII sites of the vectors.

Expression cassettes, CI (huTNF-α), CII (huTNF-α, CIII (huTNF-α), were constructed using the same strategy and techniques as making the huIL-2 and mIFN-γ expression cassettes (Chapter II and IV) except that different primers were used. For making expression cassette CI (huTNF-α), a DNA fragment containing the α-antigen promoter and signal peptide was synthesized by PCR with primers DKU76 and DKU83 from Pasteur strain BCG genomic DNA.

An XbaI site was added to the 5' end and a 10 bp sequence identical to the huTNF-α 5' encoding region was added at the 3' end. The huTNF-α encoding region was also synthesized by PCR with primers DKU68 and DKU80 from a pCD-huTNF-α (Pennica *et al.*, 1984) plasmid by adding a HindIII site at the 3' end and a 10 bp sequence identical to the α-antigen signal peptide 3' region at the 5' end. These two DNA fragments were spliced together by overlap extension by PCR using primers DKU56 and DKU69 as described (Horton *et al.*, 1989), and then digested with XbaI and HindIII and cloned into pBluescriptSK(+) (Stratagene, Cloning Systems, La Jolla, Calif. USA). After the sequence was confirmed by DNA sequencing (Sanger *et al.*, 1977), the DNA fragment containing the α-antigen-huTNF-α sequence was cloned into the XbaI and HindIII sites of pMV306 and pMV261 vectors.

Expression cassette CII (huTNF-α) was constructed from CI (huTNF-α) and another plasmid pAB26 (Stover *et al.*, 1993) which has the α-antigen gene linked to the heat shock protein (hsp60) promoter of BCG. Briefly, an XbaI-SacII fragment having the hsp60 promoter and part of the α-antigen signal peptide sequence was obtained from pAB26 and ligated to a SacII-HindIII fragment having the whole huTNF-α encoding sequence and part of the α-antigen signal peptide sequence from CI (huTNF-α), and cloned into the XbaI and HindIII sites of pMV306 and pMV261.

The expression cassette CIII (huTNF-α) was constructed by ligating the PCR-synthesized huTNF-α encoding sequence to the BamHI and HindIII sites located downstream of the hsp60 regulatory

sequences of pMV261. Briefly, the huTNF-α encoding sequence was synthesized by PCR using primers DKU69 and DKU70 from a pCD-huTNF-α plasmid (Pennica *et al.*, 1984), and then cloned into the BamHI and HindIII sites of pBluescriptSK(+) for sequencing. After the sequences were confirmed by DNA sequencing (Sanger *et al.*, 1977), the huTNF-α encoding sequence was cloned into pMV261 and then the whole expression cassette was cut out with XbaI and HindIII and cloned into pMV306.

Various expression plasmids were transformed into the BCG Pasteur strain by electroporation as described in Chapter II.

***In vitro* mutagenesis.**

To mutate the initiation codon of the hsp60-huTNF-α fusion gene from AUG to GUG, the huTNF-α expression cassette (CIII), (including the promoter and the ribosomal binding site of the hsp60 gene, the DNA encoding the first 8 amino acids of hsp60 protein and the huTNF-α encoding sequences) was first cloned into the XbaI and HindIII sites of pBluescriptSK(+) MCS. PCR was carried out by using primers T3 and DKU99 in which a T residue was changed to C. The PCR product was cloned into pBluescriptSK(+) for sequencing (Sanger *et al.*, 1977). After the sequences were confirmed, the mutated fragment was cloned into the XbaI and BamHI site of pMV261-huTNF-α to replace the original fragment.

HuTNF-α bioassay.

The bioassay of huTNF-α was carried out as described in Branch *et al.* (1991). Five X 10^4 TNF-α sensitive L-929-8 cells were added to the wells of a 96-well microtiter plate in 50µl of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% (V/V) FBS and 2 µg/ml actinomycin (Sigmal Chemical Company, USA). The plate was incubated at 37°C in 5% CO_2 atmosphere for 2 hours, then 50µl of diluted sample (prepared by sonication of the recombinant BCG cells) or recombinant huTNF-α (R&D Systems, Minneapolis) was added to each well. The plate was incubated for a further 18-24 hours, and pulsed for 4 h with 10% of tetrazolium dye MTT (Mosmann, 1983). The resulting blue to dark product was dissolved in 0.004 N HCl made in isopropanol and the color was read at 540 nm using a Titertek Multiskan MC apparatus. The amount of huTNF-α was estimated by comparing the activities with the commercial rhuTNF-α. The commercial rhuTNF-α with ED50 of 0.02-0.05 ng/ml was purchased from R&D Systems.

Detection of HuTNF-α by ELISA.

HuTNF-α in the media and cell sonicates of various rBCG cultures was detected by ELISA using a HuTNF-α ELISA kit (R&D Systems, Minneapolis, MN). The assay was carried out according to the procedures provided by the supplier. HuTNF-α in various samples was captured by a huTNF-α-specific monoclonal antibody coated onto a microtiter plate. After washing away any unbound proteins, an anti-huTNF-α polyclonal antibody-conjugated to

horseradish peroxidase was added to each well. Following one hour incubation and washing, the peroxidase substrate was added. The absorbency at OD_{450} nm was read using a Titertek Multiskan MC apparatus (Helsinki, Finland). A standard curve was made by plotting the OD_{450} vs the concentration of huTNF-α (concentration ranges from 15.6 pg/ml to 1000 pg/ml) in the standard wells. The amount of huTNF-α in various samples was estimated based on the standard curve.

SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting were carried out as described (Stover *et al.*, 1993). Recombinant BCG cells were grown to mid-logarithmic phase in Dubos media containing Kanamycin (15 μg/ml) in a 1000 ml roller bottle, and harvested by centrifugation at 4,000g. The BCG cell pellet was washed in PBS, suspended in radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50mM Tris pH 8.0) and sonicated for 30 seconds at the 50% power setting using a Branson Sonifier Cell Disrupter with a microtip. Culture lysates were analyzed by SDS-PAGE, and stained with Coomassie brilliant blue. The stained SDS-PAGE gel was scanned with a ScanJet Plus densitometer (Hewlett Packard) and quantified by using NIH Image 1.5 Software. The Western blotting was carried out using goat anti-huTNF-α neutralizing antibody (R&D Systems) and alkaline phosphatase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc.) as the secondary antibody.

Standard recombinant huTNF-α protein with molecular weight 17.5 kDa expressed in *E. coli* (R&D system) was used as a positive control.

Isolation of RNA from BCG

Total RNA from BCG Pasteur strain and various BCG recombinants was isolated as described (Patel *et al.*, 1991). Briefly, BCG cells were disrupted by sonication in 4 M guanidinium thiocyanate lysis solution containing 0.1 M β-mercaptoethanol, 0.5% sodium N-lauroylsarcosine and 25 mM sodium citrate (pH 7.0). The RNA was purified by CsCl centrifugation (Sambrook *et al.*, 1989).

Dot blotting hybridization.

The total RNA isolated from various rBCG cells was transferred to a nylon membrane by using a Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Mississauga, ON). The hybridization was carried out using huTNF-α encoding sequences as a probe and a PCR-synthesized DNA fragment of the 16S rRNA gene (Boddinghaus *et al.*, 1991) as a control. Both the huTNF-α and the 16S rRNA probes were radiolabeled (^{32}P-dCTP) by random priming (Feinberg and Vogelstein, 1984) as described in Chapter II. The specific RNAs present in each sample were quantified by scanning the autoradiograph of the dot-blots with an Imager video system (Vector Biosystems, Toronto, ON) and analyzed by NIH Image software.

3. RESULTS

Construction of recombinant BCG strains expressing mIFN-γ.

To express and secrete huTNF-α from BCG, six different plasmids pMV261-CI (huTNF-α), pMV261-CII (huTNF-α), pMV261-CIII (huTNF-α), pMV306-CI (huTNF-α), pMV306-CII (huTNF-α) and pMV306-CIII (huTNF-α) were made as described in Materials and Methods. In pMV261-CI (huTNF-α) and pMV306-CI (huTNF-α), the huTNF-α encoding sequence was under the control of the α-antigen promoter of BCG, while the expression of the huTNF-α gene was regulated by the hsp60 promoter in pMV261-CII (huTNF-α) and pMV306-CII (huTNF-α). A signal peptide sequence isolated from the naturally secreted α-antigen of BCG (Matsuo *et al.*, 1988) was inserted between the promoter and the huTNF-α encoding sequence to facilitate secretion. In these four plasmids, the huTNF-α encoding sequence was fused directly to the signal peptide sequence of the α-antigen by gene splicing of overlap extension (Horton *et al.*, 1989). In pMV261-CIII (huTNF-α) and pMV306-CIII (huTNF-α), the huTNF-α encoding sequence was inserted into the BamHI and HindIII sites of the pMV261 expression cassette. In these two plasmids, fusion proteins with 8 amino acids derived from the hsp60 protein of BCG and the BamHI site would be expressed. In plasmids pMV261-CI (huTNF-α), CII (huTNF-α) and CIII (huTNF-α), the expression cassettes were cloned into the *E. coli*/BCG shuttle plasmid vector pMV261. The pMV261 contains an *E. coli* origin of replication (oriE), a mycobacterial origin of replication (oriM), a Kanamycin resistant gene (aph) and an expression cassette, which contains 404

bp of the 5' regulatory sequences of the BCG hsp60 gene including the first six amino acids of the hsp60 protein of BCG (Stover *et al.*, 1991). In plasmids pMV306-CI (huTNF-α), CII (huTNF-α) and CIII (huTNF-α), the expression cassettes were inserted into the integrative vector pMV306. pMV306 contains an attachment (attP) site and an integrase (int) gene derived from mycobacteriophage L5, which allows the integration of the vector into the BCG genome by site-specific recombination (Stover *et al.*, 1991).

All six expression plasmids were transformed into the BCG Pasteur strain by electroporation as described in the Materials and Methods. As shown in the Table 3.2, no colonies could be obtained from the electroporation of pMV261-CI (huTNF-α) and pMV261-CII (huTNF-α) while approximately 500-1000 colonies/μg DNA could be obtained when other expression plasmids as well as the vectors were transformed into BCG.

Expression and secretion of huTNF-α from recombinant BCG.

Electroporation of all expression plasmids into BCG Pasteur strain generated 4 different recombinant BCG strains, BCG-pMV261-CIII (huTNF-α), BCG-pMV306-CI (huTNF-α), BCG-pMV306-CII (huTNF-α), BCG-pMV306-CIII (huTNF-α). The biologically functional huTNF-α in the media and cell sonicates of these BCG recombinants was measured by a bioassay based on huTNF-α's ability to lyse a mouse tumor cell line L-929-8 as described in Materials and Methods. As presented in Table 3.2, approximately 2.5 μg huTNF-α can be detected form 10 mg wet

weight of BCG-pMV261-CIII (huTNF-α) cells (generally harvested from 1 ml of a BCG culture at log growth phase), while 0.13 μg (5% of the total huTNF-α produced) huTNF-α can be detected from the supernatant of the same culture. No huTNF-α activity can be detected from the supernatants or cell sonicates of either BCG-pMV306-CI (huTNF-α) or BCG-pMV306-CII (huTNF-α), in which the huTNF-α encoding sequence was directly ligated to the promoter and signal peptide of BCG α-antigen. One possibility is that huTNF-α is expressed or secreted by these BCG recombinants, but is not functional when attached to a signal peptide. To test this possibility, I used a huTNF-α specific ELISA to detect the huTNF-α protein in the supernatants and cell sonicates of these two BCG cell cultures. As presented in the Table 3.3, approximately 0.7 ng/ml (90%) of huTNF-α was detected from the supernatant of a BCG-pMV306-CII (huTNF-α) culture while only 0.07 ng (10%) of huTNF-α was detected from the cell sonicates. BCG-pMV306-CI (huTNF-α), in which the huTNF-α encoding sequence was under the control of α-antigen signal peptide, expressed approximate 0.018 ng/ml huTNF-α in its medium. The amount of huTNF-α detected by ELISA from the cell sonicate or supernatant of BCG-pMV261-CIII (huTNF-α) is similar to that detected by bioassay (Table 3.2 and Table 3.3).

Development of huTNF-α as a reporter gene

A preliminary study showed that the development of cutaneous lesions in BALB/c mice immunized with BCG recombinant expressing huTNF-α (BCG-pMV261-CIII) was significantly reduced

compared to the controls receiving the normal BCG (The results were presented in the 95th general meeting of ASM, Washington D.C. and not shown in this thesis). Since IFN-γ-expressing rBCG showed the most promising results in protection against leishmanial infection in BALB/c mice, further *in vivo* studies concentrated on the use of mIFN-γ-expressing rBCG (see Chapter IV).

In this chapter, I will concentrate on the report of the use of huTNF-α as a reporter gene. As shown in the last section, a relatively high level of huTNF-α (about 2.5 μg/10 mg wet weight of BCG cells) was expressed intracellularly in one of the BCG recombinants BCG-pMV261-CIII (huTNF-α). Considering the availability of a highly sensitive and simple bioassay for huTNF-α as well as the lack of other suitable reporter genes in mycobacteria, I used huTNF-α as a reporter gene to study gene expression and regulation in BCG.

In the remaining section, I will concentrate on describing the use of huTNF-α as reporter gene to study gene expression in BCG. In plasmid pMV261-CIII (huTNF-α), the huTNF-α encoding sequence was inserted into the BamHI site of pMV261, which allowed a fusion protein to be expressed with eight amino acids added to the huTNF-α protein (six amino acids were from the N-terminal of hsp60 protein and two were derived from the use of the BamHI site). As shown in Figure 3.2, a dominant protein band with the expected molecular weight (18.3 kDa) (lanes b and c), which is a little larger than the standard recombinant huTNF-α protein (17.5 kDa) (lane d), was

detected from the cell sonicates of a BCG-pMV261-CIII (huTNF-α) culture by SDS-PAGE (panel A) and Western Blotting (panel B).

To develop the huTNF-α as a useful reporter gene, I found a simple way to release the huTNF-α from BCG cells into solution. By using a Branson Sonifier Cell Disrupter with a microtip at the 50% power setting, we found that 50% of the huTNF-α proteins can be released into solution by sonication for 20 seconds, and most of huTNF-α can be released by sonication for 100 seconds (Figure 3.3). Further increase of sonication time to 200 seconds did not increase the amount of huTNF-α released. Even if cell disruption was not complete, as long as the same sonication conditions were used for every sample, the same proportion of BCG cell disruption should occur, which allows comparison of levels of huTNF-α expression in different recombinant BCG cells.

In vitro mutagenesis of the initiation codon.

The first issue I addressed was the use of the initiation codon in BCG with the use of huTNF-α as a reporter gene. Unlike other prokaryotes which rarely use GUG as their initiation codon (Korak, 1985), mycobacteria may use GUG as frequently as AUG based on the open reading frames of the cloned genes. However, as the role of GUG as an initiation codon has been confirmed by comparison with a N-terminal amino acid sequence in only a few cases (Dale and Patki, 1990), the true frequency is uncertain.

To test whether GUG is efficiently used in BCG, we altered the AUG initiation codon of the BCG hsp60-huTNF-α fusion gene to GUG

by *in vitro* mutagenesis. The mutagenesis was carried out with PCR as described in Materials and Methods. To do this, we took advantage of a BamHI site near the initiation codon. With primers DKU99 and T3, AUG was changed to GUG by one round of PCR. The results of the expression are shown in Figure 3.2. We found that the pMV261-huTNF-α with GUG as the initiation codon expressed 1.8 times as much huTNF-α as the pMV261-huTNF-α with AUG as the initiation codon (shown in the lane b and c of Figure 3.2). The proteins around 50 kDa molecular weight in lane b and lane c were used to normalize the huTNF-α bands to compensate for differences in protein quantity loaded in each lane. The protein bands around 50 kDa and the huTNF-α protein bands were scanned with a ScanJet Plus densitometer (Hewlett Packard) and quantified by using NIH Image 1.5 Sofeware. The huTNF-α bioassay showed that the pMV261-huTNF-α with GUG as the initiation codon expressed approximately 6.5 μg biologically active huTNF-α/10 mg wet weight of rBCG cells, which is 2.5 times as much huTNF-α as expressed by pMV261-huTNF-α with AUG as the initiation codon.

The main bands shown in lanes b and c of Figure 3.2 are about the same size with the expected molecular weight of 18.3 kDa, which indicates that translation with both initiation codons started at the expected sites. As shown in the western blot (panel B of Figure 3.2), a faint band smaller than the standard huTNF-α protein (17.5 kDa) can be found in both lanes b and c. Since no other initiation codon is present near the N-terminal end of the huTNF-α gene, the smaller-sized proteins are likely to be the proteolytic fragments of

huTNF-α protein. When studying the secretion of the huTNF-α protein in eukaryotic cells, Perez *et al.* (1990) identified a second cleavage site, which appears to be flanked by the amino acid 12 and 13 of the mature form of huTNF-α. Removal of the first 12 amino acids from mature huTNF-α will produce a protein with a molecular weight of 16 kDa, which is the size of the truncated protein produced from our recombinant BCG. It is possible that the second cleavage site of huTNF-α protein is recognized and cleaved by some proteases in BCG.

Change of huTNF-α specific mRNA with the alteration of initiation codons.

Some studies on *E. coli* initiation codons have shown that a change of initiation codon from GUG to AUG generally resulted in increases of both protein and the gene specific mRNA (Looman and Knippenberg, 1986). To test whether the change of AUG to GUG of the hsp60-huTNF-α fusion gene in BCG would increase the huTNF-α gene specific mRNA, total RNA isolated from BCG and BCG recombinants was subjected to dot blotting and hybridization using huTNF-α DNA as a probe and the 16S rRNA as an internal control. The results are shown in the Figure 3.4. By quantifying the autoradiograph of the dot blots of various hybridized RNA samples using rRNA as a control, we found that the huTNF-α specific mRNA was increased approximately 100 percent when the initiation codon AUG was mutated to GUG.

4. DISCUSSION

In this chapter, I described the expression of huTNF-α in *M. bovis* BCG and the use of huTNF-α as a reporter gene to study the effects of initiation codon change on gene expression in BCG.

From the transformation of six huTNF-α expression plasmids (Figure 3.1) into BCG Pasteur strain, four huTNF-α–producing BCG recombinants, BCG-pMV261-CIII (huTNF-α), BCG-pMV306-CI (huTNF-α), BCG-pMV306-CII (huTNF-α) and BCG-pMV306-CIII (huTNF-α), were obtained.

The expression of huTNF-α from two BCG recombinants, BCG-pMV261-CIII (huTNF-α) and BCG-pMV306-CIII (huTNF-α), can be detected by a bioassay as well as ELISA (Table 3.2 and Table 3.3); while the expression of huTNF-α from another two BCG recombinants, BCG-pMV306-CI (huTNF-α) and BCG-pMV306-CII (huTNF-α), in which the huTNF-α encoding sequence was ligated to a signal peptide of BCG α-antigen, could only be detected by ELISA (Table 3.3). Most of the huTNF-α protein (90%) detected by ELISA was present in the supernatant of a liquid culture of BCG-pMV306-CII (huTNF-α). Only a small amount of huTNF-α (0.18 ng/ml) could be detected by ELISA from the supernatant of a BCG-pMV306-CI culture but no huTNF-α could be detected from the cell sonicates. In both of these expression plasmids, the huTNF-α encoding sequence was linked to the α-antigen signal peptide. We believe that the signal peptide of BCG α-antigen in BCG-pMV306-CI (huTNF-α) and BCG-pMV306-CI (huTNF-α) directs the secretion of huTNF-α, and huTNF-α detected from the supernatants is not due to

the release of huTNF-α from the autolysis of recombinant BCG cells. As discussed in the chapter II, the autolysis of BCG cells normally occurs after the late log growth phase. Since the supernatants for huTNF-α detection by ELISA were always harvested before late log growth phase, it is unlikely that large amount of huTNF-α was released at this stage. Moreover, most of huTNF-α (96%) expressed by another rBCG strain BCG-pMV261-CIII (huTNF-α), in which the huTNF-α gene was not linked to any signal peptide, was present in the cell sonicates.

It is not clear why both extracellular and intracellular huTNF-α expressed by the two BCG recombinants, with the huTNF-α attached directly to the signal peptide of BCG α-antigen, is not detected in the bioassay. One possible explanation is that huTNF-α bioassay is not sensitive enough to detect the huTNF-α expressed by these two BCG recombinants; another explanation is that the huTNF-α expressed by these two rBCG is not functional. I favor the second explanation, since the huTNF-α bioassay I used is very sensitive (Branch *et al.*, 1991) and should detect 7 ng/ml huTNF-α expressed by BCG-pMV306-CII (huTNF-α). The native huTNF-α exists as a homotrimer and only the trimer or dimer exhibits receptor-binding activity, and is therefore functional (Person *et al.*, 1989). It is possible that direct ligation of huTNF-α to an α-antigen signal peptide allows the secretion of huTNF-α from BCG cells, but the secreted huTNF-α is not properly cleaved or not cleaved from the signal peptide. The improperly cleaved huTNF-α might not form the proper spatial structure required for the function of huTNF-α.

The use of a reporter gene, which encodes a well-characterized and easily-assayed protein coupled to a target DNA sequence of interest, provides a powerful tool to identify and study genetic elements involved in gene expression (Slauch and Silhavy, 1991). One of the commonly used reporter genes, β-galactosidase, has been expressed in mycobacteria, but the expression was detected by western blotting using anti-β-galactosidase antibody and by inducing antibody production *in vivo* (Murray *et al.*, 1992; Stover *et al.*, 1991). For unknown reasons, this reporter gene has not been used in studying the gene expression and regulation in mycobacteria. More recently, another useful reporter gene, luciferase, has been introduced into *M. tuberculosis* by both bacteriophage and plasmid vectors to assess the metabolic activity of bacterial cells for testing the susceptibility of *M. tuberculosis* to various drugs (Cooksey *et al.*, 1993; Jacobs *et al.*, 1993). Up to now, no reporter gene has been used to study gene expression in mycobacteria. With the expression of huTNF-α in BCG, we felt that huTNF-α could serve as a reporter gene in studying the gene expression in mycobacteria. Unlike many other heterologous proteins, recombinant TNF-α remains soluble in bacterial cells (Fiers, 1991), and it can be easily released into solution by sonication for about 100 seconds. It can also be released into medium by vortexing for about 5-10 minutes in the presence of glass beads (data not shown). The TNF-α bioassay is simple to perform, and the L-929-8 cell line that we used is very sensitive to huTNF-α in the presence of 1 μg/ml of actinomycin. The bioassay can detect femtomolar levels of huTNF-α (Branch *et al.*, 1991). In addition,

many samples can be assayed at the same time in a microtiter plate. The detection of cell lysis using the MTT method is very simple and convenient to perform (Mosmann, 1983). The use of huTNF-α as a reporter gene should be generally applicable in studying gene expression and regulation in mycobacteria.

The initiation of translation in prokaryotes involves two kinds of base pairing. One is the base pairing between Shine-Dalgarno sequences located about 10 bp 5' to the initiation codon and the 3' end of 16S rRNA in the 30S ribosomal particle; the other involves base pairing between the anticodon of initiator tRNA ($tRNAf^{met}$) and the initiation codon (Ringquist *et al.*, 1992). A number of initiation factors, especially IF3 are also involved in translation initiation (Gold, 1988). Extensive studies on the initiation of translation in *E. coli* have shown that both of these base pairings play a critical role in translation initiation, and disturbance of these base pairings by mutagenesis affects the translation efficiency (Gold, 1988; Ringquist *et al.*, 1992). In *E. coli* AUG is the most abundant initiation codon, and is preferred over other initiation codons (GUG and UUG) (Gold, 1988). Direct comparison between AUG and GUG by mutagenesis showed that AUG produced higher translation than GUG (Looman and Knippenberg, 1986; Munson *et al*, 1984; Ringquist *et al.*, 1992). It was suggested that less efficient translation associated with the use of GUG in *E. coli* is due to the weaker base pairing between the GUG and the anticodon of initiator tRNA (Ringquist *et al.*, 1992), since regardless of which initiation codon was used, the same initiator tRNA ($tRNAf^{met}$) was used and the fmet is the first amino acid

incorporated (Gold, 1988). The reaction between GUG and the anticodon of $tRNAf^{met}$ involves only two base pairing while the AUG can have 3 base pairing with the anticodon of $tRNAf^{met}$. On the other hand, some highly expressed genes in *E. coli*, including several ribosomal genes and the RNA polymerase gene rpoC use GUG as their start codons (Stormo, 1986). In some studies, mRNA with either AUG or GUG as their initiation codon show the same translation efficiency (Shinedling *et al.*, 1987). All these results have been explained by assuming that the weaker interaction of the base pairing between the GUG and the anticodon of $tRNAf^{met}$ was compensated for by strong Shine-Dalgarno interactions or by other determinants within the ribosomal binding site (Ringquist *et al.*, 1992).

In contrast to *E. coli*, approximately an equal number of genes studied so far in mycobacteria use GUG as their initiation codons (Dale and Patki, 1990). This suggests that the GUG should be used as efficiently as the AUG in mycobacteria and a different mechanism in the use of the initiation codon in mycobacteria probably exists. We found that when the initiation codon AUG of the hsp60-huTNF-α fusion gene was substituted with GUG, the expression of the huTNF-α reporter proteins in BCG was increased at least 100 percent. This is the first example showing that GUG serves as a more efficient initiation codon than AUG.

Consistent with the studies on initiation codons in *E. coli* (Looman and Knippenberg, 1986), we found that accompanying the increase of huTNF-α protein, the huTNF-α specific mRNA was also increased the same amount when the initiation codon AUG was

changed to GUG. It is quite unlikely that the change of one nucleotide in the initiation codon has a direct effect on the promoter activity, since the transcription initiation site is approximately 200 bp away from the site of nucleotide alteration (Stover *et al.*, 1991). However it is still possible that a single nucleotide change at the translational initiation could have a primary effect on transcription, for example, having a positive or negative regulatory element of transcription of the hsp60 gene located on the translational initiation site.

In prokaryotes, transcription and translation are coupled together and translation can affect the rate of mRNA synthesis or the rate of mRNA degradation (Fischer *et al.*, 1985; Grundstrom and Normark, 1985; Singer and Nomura, 1985). It is possible that one nucleotide alteration in the initiation codon of the hsp60-huTNF-α fusion gene has a primary effect on the initiation of translation, and that in turn affects the mRNA synthesis rate or the rate of mRNA degradation.

How GUG serves as a more efficient initiation codon than AUG in BCG is unknown. One possibility is that there may exist another kind of initiator tRNA (called $tRNAf^{xxx}$) in BCG with an anticodon CAC in addition to the commonly used $tRNAf^{met}$ with UAC as its anticodon. The initiator $tRNAf^{met}$ would recognize the AUG initiation codon whereas the initiator $tRNAf^{xxx}$ would normally recognize the GUG initiation codon. $tRNAf^{met}$ could bind to GUG and a $tRNAf^{xxx}$ could also bind to AUG, but both bindings would be less efficient than the normal binding due to the weaker base pairing. When the AUG

was mutated to GUG in the hsp60 gene, $tRNAf^{xxx}$ would recognize the GUG more efficiently than $tRNAf^{met}$ would recognize AUG, since the G-C pairing involves three hydrogen bonds. Normally GUG in bacteria encodes valine. If there was a new type of initiation tRNA in BCG, it would be interesting to know whether the first amino acid of hsp60-huTNF-α fusion protein is valine or methionine. Protein sequencing of the fusion protein would answer this question.

Another possibility is that the ribosomal binding sequences in the hsp60 gene favor the use of GUG as the start codon, and that the change of the AUG to GUG would alter the secondary structure in the ribosomal binding region, which has been shown to affect translation efficiency (Gold, 1988).

To understand the mechanism(s) involved, it would be necessary to know whether this effect is a general phenomenon or just a special event associated with the hsp60 gene of BCG. My preliminary *in vitro* mutagenesis studies show that the α-antigen gene of BCG uses GUG more efficiently than AUG while another two genes of BCG, MPB70 and MPB64, use AUG more efficiently (data not shown). These results suggest that the efficiency of using GUG as initiation codon in BCG must be affected by many factors and is not simply the use of a new tRNA. But in contrast to the use of initiation codon in *E. coli* and other bacteria, 50% of genes we have studied so far in BCG use GUG more efficiently than AUG, which correspond to the percentage of genes in mycobacteria using GUG as their initiation codons. To identify the mechanism(s) involved, more experiments, such as cloning the initiation tRNA gene(s) from mycobacteria, could

be carried out. Initial attempts in our lab to clone the initiation tRNA gene of BCG were not successful, possibly due to the lethality to *E. coli* of the cloned DNA fragment around the initiation tRNA gene. Future attempts should try a phage-based vector cloning system to circumvent this problem.

5. BIBLIOGRAPHY

Aldovini, A., and R.A. Young. 1990. Development of a BCG recombinant vehicle for candidate AIDS vaccines. Int. Rev. Immunol. 7: 79-83.

Aldovini, A., and R.A. Young. 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature 351: 479-482.

Boddinghaus, B., T. Rogall, T. Flohr, H. Blocker, and E. Bottger. 1993. Detection and Identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. 28: 1751-1759.

Branch, D.R., A. Shah, and L.J. Guilbert. 1991. A specific and reliable bioassay for the detection of femtomolar levels of human and murine tumor necrosis factors. J. Immunol. Methods 143: 251-261.

Buell, G., and N. Panayotatos. 1986. Mechanism and practice. In: Maximizing Gene Expression, edited by Reznikoff, W. and Gold, L. Butterworth.

Burlein, J.E., C.K. Stover, S. Offutt, and M.S. Hanson. 1994. Expression of Foreign Genes in Mycobacteria. In: Tuberculosis: Pathogenesis, Protection, and Immunity, edited by Bloom, B.R. pp239-252.

Calmette, A., C. Guérin, and B. Weill-Halle. 1924. Essais de premunition par le BCG contre l'infection tuberculeuse de l'homme et des animaux. Bull. Acad. Natl. Med.(Paris) 91: 787-796.

Cooksey, R.C., J.T. Crawford, W.R. Jr. Jacobs, and T.M. Shinnick. 1993. A rapid method for screening antimicrobial agents for activities against a strain of *Mycobacterium tuberculosis* expressing firefly luciferase. Antimicrob. Agents Chemother. 37: 1348-1352.

Dale, J.W., and A. Patki. 1990. Mycobacterial gene expression and regulation. In: Molecular Biology of the Mycobacteria, Surrey University Press. pp173-198.

Feinberg, A.P., and B. Vogelstein. 1984. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137: 266-267.

Fiers, W. 1991. Tumor necrosis factor: Characterization at the molecular, cellular and in vivo level. FEBS 285: 199-212.

Fine, P.E. 1989. The BCG story: Lessons from the past and implications for the future. Rev. of Infect. Dis. 2: 353-359.

Fischer, R.F., A. Das, R. Kolter, M.E. Winkler, and C. Yanofsky. 1985. Analysis of the requirements for transcription pausing in the tryptophan operon. J. Mol. Biol. 182: 397-409.

Gold, L. 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. Ann. Rev. Biochem. 57: 199-233.

Grundstrom, T., and S. Normark. 1985. Initiation of translation makes attenuation of ampC in *E. coli* dependent on growth rate. Mol. Gen. Genet. 198: 411-415.

Jacobs, W.R.,Jr., R.G. Barletta, R. Udani, J. Chan, G. Kalkut, G. Sosne, T. Kieser, G.J. Sarkis, G.F. Hatfull, and B.R. Bloom. 1993. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. Science 260: 819-822.

Kozak, M. 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiological Reviews 47: 1-45.

Looman, A.C., and P.H. Knippenberg. 1986. Effects of GUG and AUG initiation codons on the expression of lacZ in *Escherichia coli*. FEBS 197: 315-320.

Mosmann, T.R. 1983. Rapid colorimetric assay for cellular growth and cytotoxicity assays. J. Immunol. Methods 65: 55-63.

Munson, L.M., G.S. Stormo, R.L. Niece, and W.S. Reznikoff. 1984. lacZ translation initiation mutations. J. Mol. Biol. 177: 663-683.

Murray, A., N. Winter, M. Lagranderie, D.F. Hill, J. Rauzier, J. Timm, C. Leclerc, K.M. Moriarty, M. Gheorghiu, and B. Gicquel. 1992. Expression of *Escherichia coli* β-galactosidase in *Mycobacterium bovis* BCG using an expression system isolated from

Mycobacterium paratuberculosis which induced humoral and cellular immune responses. Mol. Microbiol. 6: 3331-3342.

Patel, B., D. Banerjee, and P. Butcher. 1991. Characterization of the heat shock response in *mycobacterium bovis* BCG. J. Bacteriol. 173: 7982-7987.

Pennica, D., G.E. Nedwin, J.S. Hayflick, P.H. Seeburg, R. Derynck, M.A. Palladino, W.J. Kohr, B.B. Aggarwal, and D.V. Goeddel. 1984. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 312:,724-729.

Perez, C., I. Albert, K. DeFay, N. Zachariades, L. Gooding, and M. Kriegler. 1990. A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. Cell 63: 251-258.

Ringquist, S., S. Shinedling, D. Barrick, L. Green, J. Binkley, G.D. Stormo, and L. Gold. 1992. Translation initiation in *Escherichia coli*: sequences within the ribosome-binding site. Mol. Microbiol. 6: 1219-1229.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74: 5463.

Shinedling, S., M. Gayle, D. Pribnow, and L. Gold. 1987. Mutations affecting translation of the bacteriophage T4 rIIB gene cloned in *Escherichia coli*. Mol. Gen. Genet. 207: 224-232.

Singer, P., and M. Nomura. 1985. Stability of ribosomal protein mRNA and translational feedback regulation in *Escherichia coli*. Mol. Gen. Genet. 199: 543-546.

Slauch, J.M., and T Silhavy, J. 1991. Genetic fusion as experimental tools. Meth. Enzymol. 204: 213-248.

Stormo, G. 1986. Translational initiation. In: Maximizing Gene Expression, edited by Reznikoff, W. and Gold, L. Butterworths. pp195-224.

Stover, C.K., G.P. Bansal, M.S. Hanson, J.E. Burlein, S.R. Palaszynski, J.F. Young, S. Koenig, D.B. Young, A. Sadzine, and A.G. Barbour. 1993. Protective immunity elicited by recombinant Bacille Calmette-Guérin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate lyme disease vaccine. J. Exp. Med. 178: 197-209.

Stover, C.K., V.F. De la Cruz, T.R. Fuerst, J.E. Burlein, L.A. Benson, L.T. Bennett, G.P. Bansal, J.F. Young, M.H. Lee, G.F. Hatfull, S.B. Snapper, R.G. Barletta, W.R. Jacobs,Jr., and B.R. Bloom. 1991. New use of BCG for recombinant vaccines. Nature 351: 456-460.

Winter, N., M. Lagranderie, J. Rauzier, J. Timm, C. Leclerc, B. Guy, M.P. Kieny, M. Gheorghiu, and B. Gicquel. 1991. Expression of heterologous genes in *Mycobacterium bovis* BCG: induction of a cellular response against HIV-1 Nef protein. Gene 109: 47-54.

Table 3.1 Primers used for constructing the huTNF-α expression plasmids

DKU68: GTTCTaagcttGGGTTCCGAC[1]
DKU71: CTAggatccGGTCAGATCATCTTCTCG[2]
DKU76: GGTctagacTTTCGCCCGAATCGA[3]
DKU80: ccgcgggcgcgGTCAGATCATCTTCTCGAAC[4]
DKU83: gatgatctgacCGCGCCCGCGGTTGCCGCTC[5]
DKU99: CTggatccGCAATTGTCTTGGCCACTGCGAA[6]
T3: ATTTAACCCTCACTAAAG

Notes:

1. Lower case letters indicate the added HindIII site
2. Lower case letters indicate the added BamHI site
3. Lower case letters indicate the added XbaI site
4. Lower case letters indicate sequence identical to α-antigen signal peptide 3' sequence
5. Lower case letters indicate the sequence identical to the huTNF-α 5' encoding sequence
6. Lower case letters indicate the added HindIII site and CAC indicates the site for changing the initiation codon

Table 3.2 Expression of huTNF-α in BCG detected by bioassay

	BCG Colonies	huTNF-α (ng)[a] in Supernatants	huTNF-α (ng)[b] in Cell Sonicates
pMV261-CI(huTNF-α)	0		
pMV261-CII(huTNF-α)	0		
pMV261-CIII(huTNF-α)	500-1000	130 (5%)	2,500 (95%)
pMV306-CI(huTNF-α)	500-1000	0	0
pMV306-CII(huTNF-α)	500-1000	0	0
pMV306-CIII(huTNF-α)	500-1000	0.8	4

Notes

a. HuTNF-α detected with bioassay is from 1 ml of a saturated culture of various recombinant BCG (rBCG). All samples were done in triplicate, and the stardard error of means were all less than 5%.

b. HuTNF-α de'ected with bioassay is from the sonicates of 1 ml of a saturated culture of various rBCG and 1 ml saturated growth of BCG cells generally weighs 10 mg.

Table 3.3 Expression of huTNF-α in BCG detected by ELISA

	huTNF-α (ng)[a] in Supernatants	huTNF-α (ng)[b] in Cell Sonicates
pMV261-CIII(huTNF-α)	91 (4%)	2700 (96%)
pMV306-CI(huTNF-α)	0.18	0
pMV306-CII(huTNF-α)	0.70 (90%)	0.070 (10%)

Notes:

a. HuTNF-α detected by ELISA is from 1 ml of log growth culture of various rBCG. All samples were done in triplicate, and the stardard error of means were all less than 5%.

b. HuTNF-α detected by ELISA is from the sonicates of 1 ml of saturated growth culture of various rBCG and 1 ml saturated growth of BCG cells generally weigh 10 mg.

Figure 3.1 Plasmids used for expressing huTNF-α in BCG.

A. Two *E. coli*/BCG shuttle plasmid vectors pMV306 and pMV261 used for making expression plasmids. Explanation for these plasmid vectors can be found in the Results.

B. Three expression cassettes, CI (huTNF-α), CII (huTNF-α) and CIII (huTNF-α), constructed for expressing huTNF-α. Explanation for making these constructs can be found in the Materials and Methods.

Insertion of three expression cassettes into two vectors generates six expression plasmids, pMV261-CI (huTNF-α), pMV261-CII (huTNF-α), pMV261-CIII (huTNF-α), pMV306-CI (huTNF-α), pMV306-CII (huTNF-α), pMV306-CIII (huTNF-α). All three expression cassettes were inserted into the XbaI and HindIII sites of the vectors.

The restriction enzymes shown in the map are: K, KpnI; P, PstI; E, EcoRI; H, HindIII; B, BamHI; Bg, BlgII; N, NotI; S, SalI; X, XbaI.

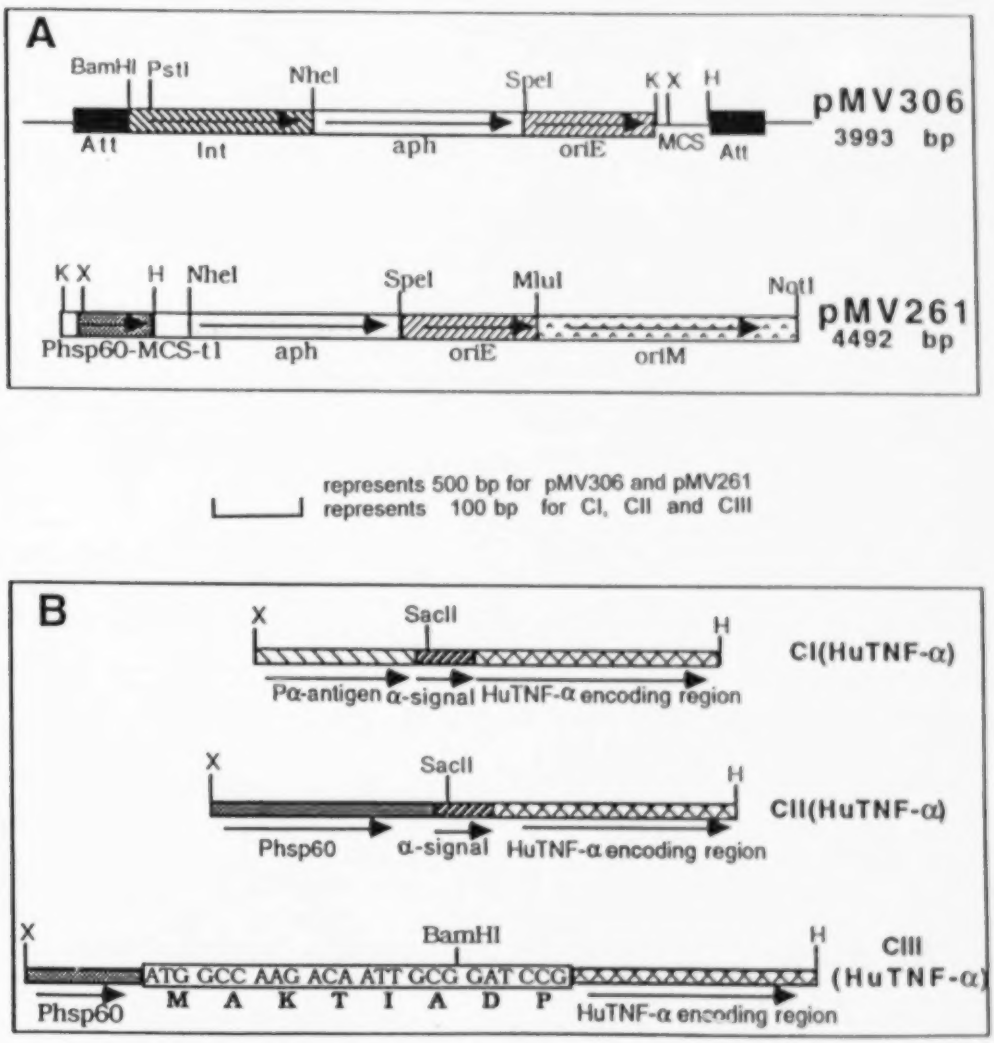

A
BamHI
PstI
NheI
SpeI
K X
H
pMV306
3993 bp
Att
Int
aph
oriE
MCS
Att
K X
H
NheI
SpeI
MluI
NotI
pMV261
4492 bp
Phsp60-MCS-t1
aph
oriE
oriM
represents 500 bp for pMV306 and pMV261
represents 100 bp for CI, CII and CIII
B
X
SacII
H
CI(HuTNF-α)
Pα-antigen
α-signal
HuTNF-α encoding region
X
SacII
H
CII(HuTNF-α)
Phsp60
α-signal
HuTNF-α encoding region
X
BamHI
H
CIII
(HuTNF-α)
ATG GCC AAG ACA ATT GCG GAT CCG
Phsp60
M A K T I A D P
HuTNF-α encoding region

Figure 3.2 Coomassie brilliant blue-stained SDS-PAGE (Panel A) and Western blot (Panel B) analysis of huTNF-α proteins expressed in various BCG recombinants. Approximately 2 μg total protein was loaded in each lane.

Lanes: a, BCG-pMV261 vector control; b, BCG-pMV261-huTNF-α with AUG as the initiation codon; c, BCG-pMV261-huTNF-α with GUG as the initiation codon; d, Commercial rhuTNF-α (R&D Systems) control. Arrows indicate the bands corresponding to the expressed huTNF-α protein with the expected molecular weights and stars indicate the control huTNF-α protein bands.

The protein markers used are: Phosphorylase B (97.4 kDa), Bovine Serum Albumin (66.2 kDa), Glutamate Dehydrogenase (55.0 kDa), Ovalbumin (42.7 kDa), Aldolase (40.0 kDa), Carbonic Anhydrase (31.0 kDa), Soybean Trypsin Inhibitor (21.5 kDa), Lysozyme (14.4 kDa).

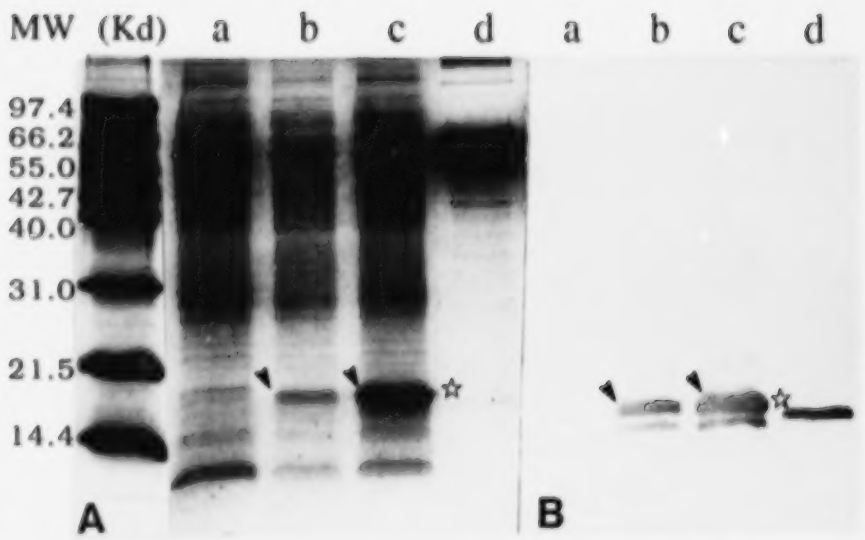

MW (Kd)
a
b
c
d
a
b
c
d
97.4
66.2
55.0
42.7
40.0
31.0
21.5
14.4
A
B

Figure 3.3 Release of biologically functional huTNF-α from rBCG cells by different sonication times. HuTNF-α activity was determined by using the huTNF-α sensitive cell line L-929-8 as described in Materials and Methods. The amount of huTNF-α released with different sonication times was estimated by comparison with the rHuTNF-α (R&D systems). All samples were done in triplicate, and the standard error of means were all less than 5%.

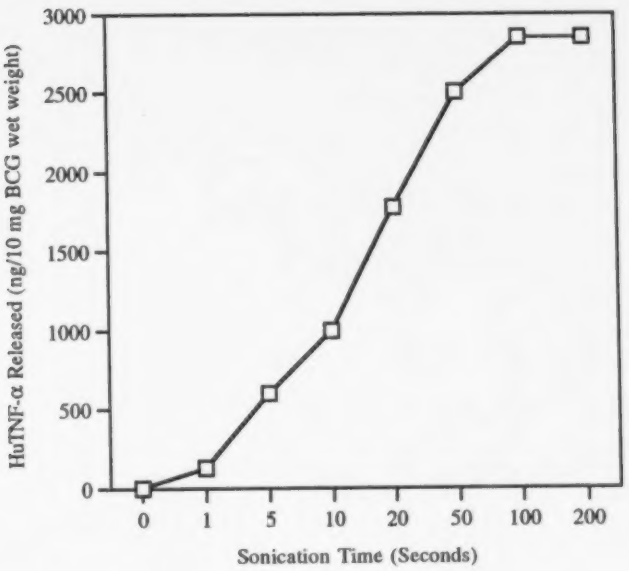
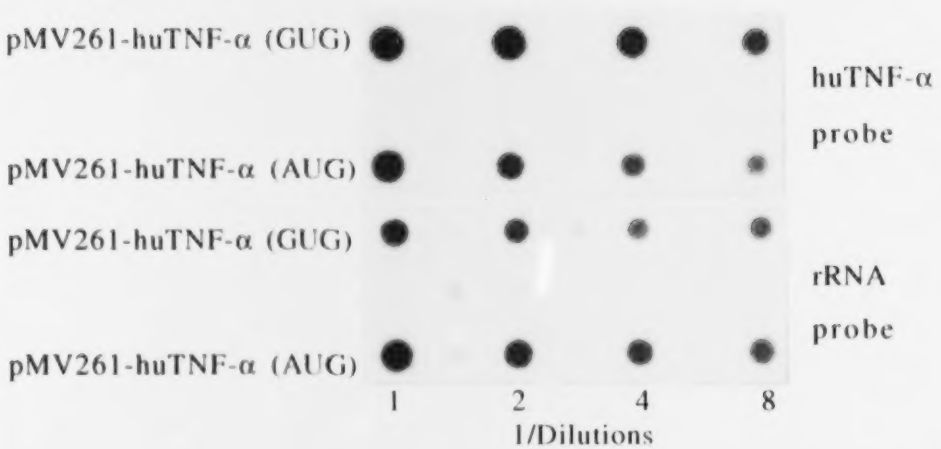

HuTNF-α Released (ng/10 mg BCG wet weight)
3000
2500
2000
1500
1000
500
0
0
1
5
10
20
50
100
200
Sonication Time (Seconds)

Figure 3.4 Dot blot hybridization analysis of RNA isolated from recombinant, BCG-pMV261-HuTNF-α with AUG or GUG as the initiation codon. HuTNF-α specific mRNA was detected using a ^{32}P-labeled huTNF-α encoding sequence, while the rRNA controls were detected using a PCR-synthesized DNA fragment of 16S rRNA as a probe.


pMV261-huTNF-α (GUG)
pMV261-huTNF-α (AUG)
pMV261-huTNF-α (GUG)
pMV261-huTNF-α (AUG)
huTNF-α probe
rRNA probe
1
2
4
8
1/Dilutions

Chapter IV

Immunization with mIFN-γ-secreting rBCG in BALB/c mice either suppresses or enhances *Leishmania major* infection depending on the time of immunization and challenge

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The information presented in this chapter is being prepared for publication. **Kong, D.**, Belosevic, M. and Kunimoto, D.Y. 1995. Protection or Exacerbation of *Leishmania major* infection in BALB/c mice by mIFN-γ-secreting rBCG is dependent on the time of infection (In preparation).

Preparation of *L. major* amastigotes for infection and estimation of *L. major* number in the infected footpads of mice were performed by Dr. M. Belosevic.

1. INTRODUCTION

The protozoa leishmania is the etiologic agent of leishmaniases, which afflicts an estimated 12 million people in 86 countries around the world (Molyneux and Ashford, 1983). Leishmania infection in mammalian hosts results in a spectrum of diseases ranging from self-healing localized cutaneous and mucocutaneous ulceration to progressive visceral dissemination (for reviews, see Sacks *et al.*, 1993; Wilson, 1993). The severity of the leishmaniasis is thought to be dependent on both the infected leishmania species and the host immune response (Ridley, 1988). Infection of different inbred mouse strains by *Leishmania major* provides a model system to study the different immune determinants on the development of the spectrum of diseases (Kellina, 1973; Behin *et al.*, Handman *et al.*, 1979). Infection of most inbred mouse strains, such as CBA, C3H and C57BL/6, with *L. major* leads to a localized cutaneous lesion that heals spontaneously. However, infection of a few inbred mouse strains, such as BALB/c, causes cutaneous and progressive, systemic disease that leads to death of the animals in about 12 weeks.

Several immunological abnormalities have been suggested by some early studies to be responsible for the extreme susceptibility of BALB/c mice to infection by *L. major*: defective macrophage responses to lymphokine activation (Nacy *et al.*, 1983), reduced capacity of lymphocytes to produce macrophage-activating lymphokines (Murray *et al.*, 1982; Sadick, 1986), development of suppressor T lymphocytes (Howard *et al.*, 1980; Titus *et al.*, 1985)

and suppression of lymphocyte function by macrophages (Scott and Farrell, 1981). These events clearly happen during infection, but it has been shown by many studies that susceptible mice do not exhibit an irreversible or general immunological defect. Susceptible BALB/c mice can be rendered resistant to *L. major* infection by some immunological manipulation such as prior sublethal irradiation, depletion of CD_4 T cells with anti-CD_4 monoclonal antibodies and Cyclosporin A treatment (Titus *et al.*, 1985; Sacks *et al.*, 1984; Scott *et al.*, 1986).

L. major is an obligate intracellular pathogen, which replicates only in the macrophage of mammalian hosts (Nacy and Diggs, 1981). Therefore, activation of macrophages to kill the infecting *L. major* is essential for the host control of infection. Many studies, including cell transfer and depletion, application of cytokines or antibodies against cytokines have indicated that CD_4 T lymphocytes as well as T cell and macrophage-secreted cytokines play a crucial role in the host's defense against *L. major* infection (for review, see Bogdan *et al.*, 1993).

Evidence accumulated in recent years clearly established that two subsets of CD_4 T cells, designated Th1 and Th2 with different patterns of lymphokine secretion, seem to play opposite roles in murine leishmaniasis (for reviews, see Bogdan *et al.*, 1993; Sher, 1992; Liew, 1989). Resistance to *L. major* infection is associated with the stimulation of Th1 T cell subsets, which produce IL-2 and IFN-γ, while susceptibility is associated with the activation of Th2 T cell subsets, which secrete IL-4, IL-5 and IL-10. The evidence includes:

1). Susceptible mice (BALB/c) infected with *L. major* show strong humoral immune responses and produce a high level of IgE, but have no delayed type hypersensitivity (DTH) reactivity; whereas infected resistant mice (CBA) show a strong DTH response, but poor antibody production (Liew, 1989; Heinzel *et al.*, 1989).

2). T cells from *L. major*-infected susceptible mice after *in vitro* stimulation with leishmanial antigens secrete significant amounts of IL-4 and IL-5, but little IFN-γ, while T cells from *L. major* resistant mice secrete significant amounts of IFN-γ but little IL-4 and IL-5 in response to leishmania antigen (Sadick *et al.*, 1986; Boom *et al.*, 1990).

3). CD4 T cells isolated from susceptible BALB/c mice express significant levels of IL-4 mRNA but little IFN-γ mRNA, whereas T cells from resistant C57/BL6 mice express substantially higher levels of IFN-γ but no IL-4 mRNA (Heinzel *et al.*, 1989; Heinzel *et al.*, 1991).

4). Protective immunity can be transferred to BALB/c mice by leishmania-specific Th1 T cell lines while the transfer of Th2 T cell lines exacerbates the nonhealing disease (Scott, 1988).

5). A single injection of monoclonal antibody against IFN-γ during the first one or two weeks after *L. major* infection can convert a resistant mouse into a susceptible one (Belosevic *et al.*, 1989; Scott, 1991).

6). *In vivo* neutralization of IL-4 in susceptible BALB/c mice at the beginning of an infection can convert the mice into a resistant phenotype (Sadick *et al.*, 1990).

The opposite roles that the Th1 and Th2 T cell subsets play during *L. major* infection can be understood in terms of the regulatory and specific effector functions exhibited by the cytokines secreted by each T cell subset. First of all, IFN-γ secreted by the Th1 T cell subset, is a major macrophage activator although the presence of other cofactors, such as IL-2 or TNF-α, are required for maximal activity of IFN-γ (Bogdan *et al.*, 1990; Liew *et al.*, 1990; Belosevic *et al.*, 1990). Macrophages stimulated *in vitro* by recombinant IFN-γ exhibit three types of activation: reduced binding of the parasites (Mosser and Handman, 1992), decreased parasite uptake (Panosian and Wyler, 1983) and enhanced intracellular killing of leishmania (Buchmuller and Mauel, 1979; Titus *et al.*, 1984). In contrast, IL-4 and IL-3, secreted by the Th2 cell subset, have been shown to inhibit the activation of macrophages (Liew *et al.*, 1989; Liew *et al.*, 1991). Secondly, cytokines secreted by each of the CD_4 subsets can downregulate the proliferation or functional activity of the opposing subset (Mosmann *et al.*, 1986; Mosmann and Moore, 1991). Thus, IFN-γ produced by Th1 cells preferentially inhibits the proliferation of Th2 lymphocytes, whereas Th2 cell-produced IL-4 and IL-10, preferentially stimulates the generation of Th2, and downregulates the production of IFN-γ and IL-2 by Th1 cells.

While the administration of antibodies to IL-4 or IFN-γ at an early stage of infection can reverse the resistant or susceptible phenotype (Belosevic *et al.*, 1989; Sadick *et al.*, 1990), neither short-term nor long-term injection of either IL-4 or IFN-γ could change the disease outcome (Sadick *et al.*, 1990; Sadick *et al.*, 1991;

Scott, 1991). This has been explained by the very short half-lives *in vivo* of IL-4 and IFN-γ compared to immunoglobulin, or that IFN-γ and IL-4 are necessary but not sufficient for the development of Th1 and Th2 responses (Sher, 1992).

Mycobacterium bovis BCG, an attenuated strain of *M. bovis*, is the vaccine used for the prevention of *M. tuberculosis* infection. BCG has also been used as a general immunostimulator. Although the mechanisms involved are still unclear, BCG has been applied in the treatment of some tumors such as bladder cancer. BCG vaccination can render BALB/c mice more resistant to *L. major* and *L. tropica* infection (Weintraub and Weinbaum, 1977; Fortier *et al.*, 1987; Stefani *et al.*, 1993). Furthermore, with the development of gene transfer and expression systems in mycobacteria, BCG has been proposed as a vaccine vehicle to express and deliver foreign antigens. It has been shown by a recent report that expression of a surface protein gp63 of *L. major* by rBCG injected into BALB/c and CBA/J mice can induce some level of protection against *L. major* and *L. mexican* infection (Connell *et al.*, 1993). Recently, human and mouse IL-2 have been introduced and expressed in BCG (O'donnell *et al.*, 1994; Kong and Kunimoto, 1995). It has been found that secretion of huIL-2 by rBCG can render BALB/c mice more resistant to mycobacterial infection (Kong and Kunimoto, 1995).

In this chapter, I report the expression of mouse IFN-γ in BCG and some *in vivo* studies regarding the effects of a mIFN-γ-secreting rBCG immunization on subsequent infection with *L. major* in BALB/c mice. We have found that vaccination with the mIFN-γ-secreting

rBCG can either protect or exacerbate the *L. major* infection in BALB/c mice, depending on the timing of vaccination and infection.

2. MATERIALS AND METHODS

Bacterial strains, plasmids

Bacterial strains and plasmid vectors used during this study were described in the Materials and Methods of Chapter II.

Oligonucleotide primers.

Oligonucleotide primers were purchased from the Department of Biological Sciences, University of Alberta, who synthesized using a DNA synthesizer (Applied Biosystems, Inc., Foster city, Calif.) . Different primers used in constructing the mIFN-γ expression plasmids are shown in Table 4.1.

Construction of expression plasmids.

The plasmids used in expressing mIFN-γ are shown in Fig. 4.1; the vectors, pMV261 and pMV306, are shown in panel A while three expression cassettes, CI (mIFN-γ), CII (mIFN-γ) and CIII (mIFN-γ), are shown in panel B. Insertion of the three expression cassettes into the multiple cloning sites (MCS) of vector pMV261 and pMV306 generates six different plasmids. All expression cassettes were inserted into the XbaI and HindIII sites of the vectors.

Expression cassettes, CI (mIFN-γ), CII (mIFN-γ), CIII (mIFN-γ), were constructed using the same strategy and techniques used to make the huIL-2 and huTNF-α expression cassettes (Chapter II and

III) except that primers used for PCR were different. For making expression cassette CI (mIFN-γ), a DNA fragment containing the α-antigen promoter and signal peptide was synthesized by PCR (with primers DKU76 and DKU81) from BCG genomic DNA. An XbaI site was added at the 5' end and a 10bp sequence identical to the mIFN-γ 5' encoding sequence was added at the 3' end. The mIFN-γ encoding sequence was also synthesized by PCR with primers DKU69 and DKU82 from a pCD-mIFN-γ (Gary and Goeddel, 1983) plasmid by adding a HindIII site at its 3' end and a 11 BP sequence identical to the α-antigen signal peptide 3' region at its 5' end. These two DNA fragments were spliced together by overlap extension by PCR using primers DKU56 and DKU69 as described (Horton *et al.*, 1989), and then digested with XbaI and HindIII and cloned into pBluescriptSK(+) (Stratagene, Cloning Systems, LA Jolla, Calif. USA). After the sequence was confirmed by DNA sequencing (Sanger *et al.*, 1977), the DNA fragment containing the α-antigen-mIFN-γ sequence was cloned into the XbaI and HindIII sites of the pMV306 and pMV261 vectors.

Expression cassette CII (mIFN-γ) was constructed from CI (mIFN-γ) and another plasmid pAB26 (Stover *et al.*, 1993) which has the α-antigen gene linked to the heat shock protein 60 (hsp60) promoter of BCG. Briefly, an XbaI-SacII fragment having the hsp60 promoter and part of the α-antigen signal peptide sequence was obtained from pAB26 and ligated to a SacII-HindIII fragment having the whole mIFN-γ encoding sequence and part of the α-antigen signal peptide sequence from CI (mIFN-γ), and cloned into the XbaI-HindIII sites of pMV306 and pMV261.

The expression cassette CIII (mIFN-γ) was constructed by ligating the PCR-synthesized mIFN-γ encoding sequence to the BamHI and HindIII sites located downstream of the hsp60 regulatory sequences of pMV261. Briefly, the mIFN-γ encoding sequence was synthesized by PCR using primers DKU69 and DKU70 from a pCD-mIFN-γ plasmid (Gray and Goeddel, 1983), and then cloned into the BamHI and HindIII sites of pBluescriptSK(+) for sequencing. After the sequences were confirmed by DNA sequencing (Sanger *et al.*, 1977), the mIFN-γ encoding sequence was cloned into pMV261 and then the whole expression cassette was cut out with XbaI and HindIII and cloned into pMV306.

The various expression plasmids were transformed into the BCG Pasteur strain by electroporation as described in Chapter II.

Detection of mIFN-γ by ELISA

mIFN-γ present in the supernatants and cell sonicates of various recombinant BCG strains was measured by ELISA using a mouse IFN-γ ELISA kit (Genzyme, Cambridge, MA USA). The assay was carried out according to the procedures provided by the company. Briefly, mIFN-γ in various samples was captured by the first antibody (monoclonal anti-murine IFN-γ) coated on to a 96-well plate. After the plate was washed with washing buffer (PBS + 0.05% Tween 20), the secondary antibody (polyclonal goat anti-murine IFN-γ) was added. The plate was incubated at room temperature for two hours, washed, and then the third antibody (polyclonal donkey anti-goat-perioxidase) was added. Following one hour incubation

and washing, the peroxidase substrate was added. The absorbance at 450 mm was read using a Titertek Multiskan MC apparatus. A standard curve was made by assaying the provided standard mIFN-γ and the concentration of mIFN-γ in various samples was estimated based on the standard curve.

Bioassay of mIFN-γ

The biological activity of recombinant mIFN-γ expressed by rBCG was assessed based on IFN-γ's ability to stimulate a macrophage cell line J774.A1 (ATCC, Rockville, MD) to produce NO_2^- in the presence of lipopolysaccharide (LPS) (Difco Laboratories, Detroit, MI) (Cunha *et al.*, 1993). One hundred thousand J774.A1 cells suspended in 100 µl DMEM-10% FBS medium were seeded in a 96-well flat-bottom plate (Nunc, Denmark), and then 100 µl diluted samples prepared from the supernatants or cell sonicates of various rBCG strains were added to each well. The same volume of 200 u/ml of recombinant mIFN-γ (R&D system) was used as a positive control. After the addition of 50 µl LPS (2 µg/ml), the plate was incubated at 37°C in 5% CO_2 atmosphere. After 3 days incubation, 50 µl solution from each well was transferred to a new 96-well plate and 100 µl of 1% sulfanilamide (Sigma) and 100 µl of 0.1% N-1-naphthyl-ethylenediamine (Sigma) dissolved in 2.5% H_3PO_4 were added to each well. The resulting pink to dark color was read at 540 nm using a Titertek Multiskan MC apparatus (Helsink, Finland). The NO_2^- was quantified by comparison to different concentrations of

$NaNO_2$ as a standard and expressed as NO_2^- concentration (μM) in a 250 μl bioassay.

Leishmania major

Amastigotes of *L. major* (NIH 173 strain) were maintained by monthly passage in footpads of BALB/c mice (i.e. by subcutaneous inoculation of 5 x 10^5 amastigotes suspended in 50 μl PBS). After three to four weeks, the swollen footpads were cut off and the amastigote forms were released into DMEM medium from infected macrophages by homogenization in a Ten Broek tissue homogenizer.

The number of viable *L. major* amastigotes was determined using fluorescein diacetate (FDA)/ethidium bromide (EtBr) staining method as described (Jackson *et al.*, 1985). Briefly, 50 μl of an *L. major* amastigote suspension was mixed with 25 μl of FDA (5 mg/ml in acetone) and 25 μl of EB (25 mg/ml). The mixture was left at room temperature for two minutes, and then the amastigotes were counted in a hemocytometer using an optical fluorescein microscope. The esterase present in the live *L. major* cells converts nonfluorescent FDA to fluorescein, which makes the live cells show yellow-green under the fluorescent illumination. On the other hand, the dead cells can not convert the nonfluorescent FDA to fluorescein and unlike living cells, are rapidly penetrated by EtBr. Binding of EtBr to nucleic acids causes dead cells under illumination to fluoresce red. After counting, the amastigotes were adjusted to 2 x 10^7 viable parasites/ml in PBS for inoculation.

Inoculation of BCG and *L. major*

The methods for growing BCG cells and estimating their number have been described in Chapter II. 10^6 CFU BCG Pasteur or recombinant BCG-mIFN-γ (pMV261-CIII) suspended in 0.1 ml PBS were inoculated intraperitoneally into each mouse. 0.1 ml of PBS was injected into the mice in the control groups. After one, two or three weeks of BCG vaccination, 0.05 ml of parasite suspension containing 10^6 viable *L. major* amastigotes was inoculated into the left footpad of each mouse, and the right foot served as the uninoculated control. Five to ten mice were used in each experimental group.

Assessment of infection

Development of cutaneous disease was assessed weekly by estimating the difference of footpad thickness between the infected left foot and the uninfected right foot. The thickness of footpad was measured using a dial thickness gauge. At the time of sacrifice, the infected footpads were cut off and put into 5 ml of PBS solution and then homogenized. The number of amastigotes in each infected footpad were enumerated as described above.

Systemic disease was assessed at 4 weeks after infection. spleens were removed from the sacrificed mice, and touched to the surface of a slide. The spleen touch slides were stained by Diff-Quick (Dade Diagnostics, Aquado, PR) as described (Belosevic *et al.*, 1989). The number of amastigotes per 1000 nucleated cells were counted.

Differences among experimental groups were evaluated by Student's t test.

3. RESULTS

Construction of recombinant BCG strains expressing mIFN-γ.

To express and secrete mIFN-γ from BCG, six different plasmids pMV261-CI (mIFN-γ), pMV261-CII (mIFN-γ), pMV261-CIII (mIFN-γ), pMV306-CI (mIFN-γ), pMV306-CII (mIFN-γ) and pMV306-CIII (mIFN-γ) were made as described in Materials and Methods and shown in Figure 4.1. In pMV261-CI (mIFN-γ) and pMV306-CI (mIFN-γ), the mIFN-γ encoding sequence was under the control of the α-antigen promoter of BCG, while the expression of mIFN-γ gene was regulated by the hsp60 promoter in pMV261-CII (mIFN-γ) and pMV306-CII (mIFN-γ). A signal peptide sequence isolated from the naturally secreted α-antigen of BCG (Matsuo *et al.*, 1988) was inserted between the promoter and the mIFN-γ encoding sequence to facilitate secretion. In these four plasmids, the mIFN-γ encoding sequence was fused directly to the signal peptide sequence of the α-antigen by gene splicing by overlap extension (Horton *et al.*, 1989). In pMV261-CIII (mIFN-γ) and pMV306-CIII (mIFN-γ), the mIFN-γ encoding sequence was inserted into the BamHI and HindIII sites of the pMV261 expression cassette. In these plasmids, fusion proteins with 8 amino acids derived from the hsp60 protein of BCG and the BamHI site would be expressed. In plasmids pMV261-CI (mIFN-γ), CII (mIFN-γ) and CIII (mIFN-γ), the expression cassettes were cloned into the *E. coli*/BCG shuttle plasmid vector pMV261. The pMV261

contains an *E. coli* origin of replication (oriE), a mycobacterial origin of replication (oriM), a Kanamycin resistant gene (aph) and an expression cassette, which contains 404 bp of the 5' regulatory sequences of the BCG hsp60 gene including the first six amino acids of the hsp60 protein of BCG. In plasmids pMV306-CI (mIFN-γ), CII (mIFN-γ) and CIII (mIFN-γ), the expression cassettes were inserted into the integrative vector pMV306. pMV306 contains an attachment (attP) site and an integrase (int) gene derived from mycobacteriophage L5, which allows the integration of the vector into the BCG genome by site-specific recombination (Stover *et al.*, 1991).

All six expression plasmids were transformed into the BCG Pasteur strain by electroporation as described in the Materials and Methods. As shown in Table 4.2, no colonies could be obtained from the electroporation of pMV261-CII (mIFN-γ) and pMV261-CIII (mIFN-γ) while approximately 500-1000 colonies/μg DNA could be obtained when the other expression plasmids as well as the vector controls were transformed into BCG.

Expression and secretion of mIFN-γ from recombinant BCG.

Electroporation of the expression plasmids into the BCG Pasteur strain generates 4 different recombinant BCG strains, BCG-pMV261-CI (mIFN-γ), BCG-pMV306-CI (mIFN-γ), BCG-pMV306-CII (mIFN-γ), BCG-pMV306-CIII (mIFN-γ). The amounts of expressed mIFN-γ protein in the media and the cell sonicates of these recombinants were measured by mIFN-γ specific ELISA as described

in Materials and Methods. As presented in Table 4.2, most of the mIFN-γ (76%) produced by BCG-pMV261-CI (mIFN-γ), in which the mIFN-γ encoding sequence was directly ligated to the α-antigen promoter and signal peptide of BCG, was detected in the supernatant. On the other hand, most of mIFN-γ (86%) produced by BCG-pMV306-CIII (mIFN-γ), in which the mIFN-γ gene was not linked to a signal peptide sequence, was present in the cell sonicates. No mIFN-γ could be detected from either the supernatant or the cell sonicate of BCG-pMV306-CII (mIFN-γ).

To test whether the mIFN-γ detected by ELISA had any biological activity, we assayed the capacity of mIFN-γ present in the supernatants or cell sonicates of various BCG recombinants to stimulate a macrophage cell line J774.1 A to produce NO_2^-. As a potent macrophage activator, mIFN-γ has been found to stimulate J774.1A to secrete large amount of NO_2^- in the presence of a low concentration of LPS, which presumably provides a co-signal (Cunha *et al.*, 1993). The concentration of NO_2^- was measured by the Griess reaction (Green *et al.*, 1982) as described in Materials and Methods. As shown in Figure 4.2A, the concentration of standard NO_2^- is linear to the OD_{450} of the Griess reaction ($y = 0.004\ x + 0.006$). The concentration of NO_2^- in various samples can be obtained using the following formula: NO_2^- (μM) = (y-0.006)/0.004 (y is the OD_{450}). The concentration of mIFN-γ in various samples was estimated by comparison with the commercial rmIFN-γ (R&D Systems). A significant amount of mIFN-γ activity (approximately 100 u/ml) can be detected from the sonicate of BCG-pMV261-CI (mIFN-γ) and from

the sonicate of BCG-pMV306-CIII (mIFN-γ) (approximately 150 u /ml) as shown in Figure 4.2B, while no activity can be detected from the supernatants of both BCG recombinants (data not shown).

One likely explanation for the absence of activity is that the mIFN-γ protein released from the BCG recombinants is not stable in the Dubos media. To test this hypothesis, commercial rmIFN-γ (R&D Systems) was added to Dubos-ADC media to a concentration of 200 u/ml. This mIFN-γ containing media was put into a 1000 ml rolling bottle and kept at 37°C for two weeks. At different time intervals, 1 ml of media was removed from the culture and stored at -80C. The activity of mIFN-γ in the media removed at different times was assayed. As shown in the Figure 4.3, about 50% activity of mIFN-γ was lost during the first day of incubation at 37°C, and after 2 weeks incubation, more than 98% of the activity was lost. The loss of mIFN-γ activity in the media is not associated with the growth of BCG, since inoculation of mIFN-γ in a BCG culture produced the same results as when mIFN-γ was inoculated in media alone (data not shown).

Protective effects of mIFN-γ-secreting rBCG

Since only the BCG-pMV261-CI (mIFN-γ) recombinant, hereafter called BCG-mIFN-γ, secretes most of mIFN-γ into medium, we did *in vivo* studies only using this BCG recombinant. To test whether this mIFN-γ-secreting rBCG could render BALB/c mice more resistant to *L. major* infection, we inoculated a group of BALB/c mice i.p. with 10^6 CFU BCG-mIFN-γ and two other groups of mice with 10^6

CFU BCG Pasteur strain and PBS respectively as controls. Two weeks after the BCG vaccination, 10^6 amastigotes of *L. major* were injected subcutaneously into the left footpad of each mouse. The development of cutaneous disease was evaluated by the difference of footpad thickness between the infected left foot and noninfected right foot, and also by the parasite load in the infected footpad. It has been shown that the increase in the thickness of the infected footpad reflects the lesion size and correlates with the histopathologic stage of infection (Mauel *et al.*, 1975). As shown in the panel A of Figure 4.4., the footpads of mice immunized with BCG-mIFN-γ are significant smaller than those of mice immunized with the same amount of BCG ($p < 0.05$) and those of PBS control mice ($p = 0.001$) four weeks after the infection. The parasite load in the footpads of mice immunized with BCG-mIFN-γ is 1.6 times less than those of mice vaccinated with the same amount of BCG and 5 times less than those of PBS control mice.

Protection or exacerbation: Time dependence

Many studies have indicated that IFN-γ is required for host resistance to intracellular pathogens such as *L. major* (Sadick *et al.*, 1990) and *Listeria monocytogenes* (Metcalf and Campbell, 1994), but only during the early stage of infection. To test whether the protection of *L. major* infection by our mIFN-γ-secreting BCG could be affected by the time interval between BCG vaccination and *L. major* infection, we infected the BALB/c mice one, two or three weeks after the BCG-mIFN-γ or BCG vaccination.

As shown in Figure 4.4 (two weeks), Figure 4.5 (one week) and Figure 4.6 (three weeks), BCG immunization in all three infection courses shows some protection compared to the PBS control, as judged by both footpad thickness and the parasite load in the infected footpads. Infection of mice one week (Figure 4.5) or two weeks (see above) with the same amount of *L. major* after the BCG-mIFN-γ vaccination shows protection compared to both BCG and PBS controls. In mice infected one week after the BCG-mIFN-γ inoculation, the lesion size is significantly smaller than that in mice inoculated with BCG ($p < 0.05$) or PBS ($p = 0.01$) (Figure 4.5 A). The average parasite load per infected footpad from mice pretreated with BCG-mIFN-γ is 12 times less than that of mice pretreated with BCG and 60 times less than that of PBS control mice.

On the other hand, mice infected with *L. major* three weeks after the BCG-mIFN-γ immunization shows exacerbation of cutaneous infection after 4 weeks (Figure 4.6). The footpad thickness of mice immunized with BCG-mIFN-γ is significantly greater than that of PBS control mice ($p < 0.05$) as well as mice immunized with BCG ($p < 0.05$). The average parasite number in the footpads of mice inoculated with BCG-mIFN-γ is 4 times more than that of PBS control mice and 70 times more than that of mice inoculated with the same amount of BCG. When following the development of lesion size in infected mice, mIFN-γ-BCG immunization shows significant protection compared to BCG and PBS in the first two weeks of infection (Figure 4.6A). One week after the *L major* infection, the footpad thickness in the mice immunized with mIFN-γ-BCG is significantly smaller than that in the

mice immunized with BCG ($p = 0.0001$) and PBS ($P < 0.0001$). After two weeks infection, the footpad thickness in the mice inoculated with mIFN-γ-BCG is also significantly smaller than that in the mice immunized with BCG ($p = 0.002$) and PBS ($P = 0.0007$). Beginning from the third week of infection, the footpad thickness in the mice immunized with mIFN-γ-BCG showed no significant difference compared to the mice immunized with PBS ($p = 0.30$) but was significantly larger than that in the mice immunized with BCG ($P < 0.0001$). It seems that there is a time point at which the infection of *L. major* in the mice pre-inoculated with mIFN-γ-BCG was exacerbated.

4. DISCUSSION

In this chapter, I described the expression of mIFN-γ in *M. bovis* BCG and some in *vivo* studies concerning the effects of a mIFN-γ-producing BCG recombinant strain, BCG-pMV261-CI (mIFN-γ), on the subsequent infection with *L. major* in BALB/c mice.

From the transformation of six BCG/*E. coli* shuttle mIFN-γ expression plasmids (Figure 4.1) into BCG Pasteur strain, two mIFN-γ-producing BCG recombinants, BCG-pMV261-CI (mIFN-γ) and BCG-pMV261-CI (mIFN-γ), were obtained. The expression of mIFN-γ from these two BCG recombinants was first detected by ELISA. Most of the mIFN-γ (76%) was present in the supernatant of a liquid culture of BCG-pMV261-CI (mIFN-γ), in which the mIFN-γ encoding sequence was linked to the α-antigen signal peptide. We believe that the signal peptide of the α-antigen in BCG-pMV261-CI (mIFN-γ)

facilitates the secretion of mIFN-γ and the mIFN-γ detected in the supernatant is not due to the release of mIFN-γ from the autolysis of recombinant BCG cells. As discussed in the chapter II, the autolysis of BCG cells normally occurs after the late log growth phase. Since the supernatant for mIFN-γ detection by ELISA was always harvested before late log growth phase, it is unlikely that a large amount of mIFN-γ was released at this stage. Moreover, for another rBCG strain BCG-pMV306-CIII (mIFN-γ), in which the mIFN-γ gene was not linked to any signal peptide, most of the mIFN-γ (86%) was detected in the cell sonicates.

Even though a significant amount of mIFN-γ was detected from the supernatant of both recombinant BCG cultures by ELISA, no activity can be detected in a biological assay based on IFN-γ's ability to stimulate the production of NO_2^- from the macrophage cell line J774.A1. By inoculating an amount of commercial rIFN-γ into Dubos-ADC media, it was found that the mIFN-γ is not stable at 37°C. This finding is a surprise. Even though it has been found that IFN-γ is not stable at temperature above 56°C and at pH values less than 4.0 and great than 9.0 (Mulkerrin *et al.*, 1989), mIFN-γ should be stable at 37°C in the Dubos medium (pH is around 7). Murine IFN-γ exists as a noncovalent homodimer, and only the dimer can bind to the receptor, therefore, exhibit biological activity (Greenlund *et al.*, 1992). It is possible that some components of the Dubos media affect the formation of mIFN-γ dimer or trimer, therefore, affect its function. To test this hypothesis, it would be interesting to know whether mIFN-γ is stable in other media such as LB and DMEM.

Theoretically, mIFN-γ secreted from BCG recombinants *in vivo* should function in physiological conditions.

The three most commonly used methods for assaying the biological activities of mIFN-γ are based on IFN-γ's ability to induce antiviral activity in cells (Rubenstein *et al.*, 1981), induce MHC class II antigen expression on susceptible cells (King and Jones, 1983) and activate macrophages for tumoricidal or microbicidal activities (Buchmeier and Schreiber, 1985; Pfizenmaier *et al.*, 1985). Since these methods were not available in our laboratory, we adapted a simple method based on IFN-γ's ability to stimulate the production of NO_2^- by a macrophage cell line J774.1A in the presence of LPS (Cunha *et al.*, 1993). The measurement of NO_2^- is simple to perform and the concentration of NO_2^- is linear to the OD_{450} of the Griess reaction. By comparing the results to the standard mIFN-γ, the biological activities of mIFN-γ in various samples were estimated.

It has been demonstrated by many studies that pretreatment of BALB/c mice with BCG can render this highly susceptible mouse more resistant to *L. major* infection (Weintraub and Weinbaum, 1977; Fortier *et al.*, 1987; Stefani *et al.*, 1993). But treatment of BALB/c mice or other animals with BCG is not always beneficial to the animals infected by *L. major* or other leishmanial species. The effects of BCG treatment depend on many factors, such as the BCG substrains, the dose and the route of administration (For a review, see Frommel and Lagrange, 1989). In some studies, pretreatment with BCG or treatment with BCG after the infection exacerbated systemic and/or cutaneous leishmanial infections. For example,

subcutaneous injection of a BCG-*L. enriettii* mixture into a BCG pretreated-guinea-pig accelerated the development of metastases (Behin *et al.*, 1977). *L. donovani* infection of a BCG-pretreated hamster died earlier than the untreated controls (Jarecki-Black *et al.*, 1984). BCG treatment of C3H mice with established *L. mexicana* infection resulted in accelerated metastatic spread of lesions (Grimaldi *et al.*, 1980). It has been shown by a recent experiment (Stefani *et al.*, 1993) that low dose (5 X 10^4 BCG) sensitized BALB/c mice infected subcutaneously with a mixture of BCG and *L. major* led to the development of more severe leishmanial lesions. Although the mechanism(s) accounting for the exacerbation of leishmanial infections under the circumstances described above are unknown, all these studies show that host immune responses to infectious parasites are a complex process, and that administration of an immuostimulating agent, such as BCG, does not always help to control the diseases; under some conditions, it is harmful to the host immune system.

In our studies, we confirmed that BCG vaccination of BALB/c mice could reduce the severity of cutaneous infection by *L. major* as judged both by the footpad lesion size and the parasite load in the infected footpads. When studying the effects of our mIFN-γ-secreting BCG recombinant on *L. major* infection in BALB/c mice, we found that vaccination with the mIFN-γ-secreting rBCG could either protect or exacerbate the *L. major* infection depending on the timing of vaccination and infection. Vaccination of BALB/c mice with mIFN-γ-secreting BCG one or two weeks before *L. major*

infection showed significant protection from cutaneous disease, while vaccination of BALB/c with mIFN-γ-secreting BCG three weeks before *L. major* infection exacerbated the development of cutaneous disease. This finding is surprising, since it has been well established by both *in vitro* and *in vivo* studies that IFN-γ plays a crucial role in mouse resistance to *L. major* infection. Therefore mIFN-γ secreted by recombinant BCG should function *in vivo* to protect the mice from *L. major* infection.

The mechanism(s) involved in the exacerbation of *L. major* infection by our mIFN-γ-secreting BCG are unknown. *L. major* is an obligate intracellular pathogen, which replicates only in the macrophages of mammalian hosts (Nacy and Diggs, 1981). Therefore, activation of macrophages to kill the infected *L. major* is essential for the host control of infection. Any mechanism proposed to explain the effects of our mIFN-γ-secreting BCG on *L. major* infection should relate to the function of macrophages.

At an early stage of infection, the mIFN-γ secreted by rBCG could activate macrophages to kill the infected *L. major*, this can explain the early protective role of mIFN-γ-secreting BCG. It is possible that continuous exposure of macrophages to mIFN-γ expressed by recombinant BCG impairs the function of macrophages; therefore, at a late stage of infection, the macrophages could not function properly to destroy the infected *L. major*, resulting in an exacerbated effect. For example, exposure of J774A.1 macrophage cells to approximately 200 units/ml mIFN-γ *in vitro* results in the death of cells (personal observation).

An alternative explanation is that since BCG is a facultative intracellular bacterium and macrophages are the main habitat, it is possible that mIFN-γ secreted by mIFN-γ-secreting BCG was digested by macrophage enzymes, and therefore, secreted out as nonfunctional peptides. The peptides might bind to the mIFN-γ receptor and interfere with the activation of macrophages by endogenous IFN-γ. However this explanation does not account for the initial protective effect. To understand the mechanism(s) involved, the function of the macrophages from the mice vaccinated with mIFN-γ-secreting BCG should be studied at different time points (see chapter VI).

Another possibility is that although the recombinant BCG used in these studies express mIFN-γ, it is still possible that anti-mIFN-γ antibodies were developed in the mice immunized with the mIFN-γ-expressing rBCG, since the modification of protein in eukaryotes is different from that in prokaryotes, for example, the natural mIFN-γ is a glycoprotein and glycosylation generally does not occur in prokaryotes. It may be possible that development of anti-mIFN-γ antibodies at a late stage of vaccination interferes with the activation of macrophages, therefore exacerbating the *L. major* infection in the mice immunized with the mIFN-γ-expressing rBCG. To test this possibility, anti-mIFN-γ antibodies should be looked for at different times after vaccination.

It has been shown by a recent study that simultaneous stimulation with rIFN-γ and infection with *M. bovis* can induce murine bone marrow-derived macrophages to produce IL-10 (Flesch

et al., 1994) *in vitro*. IL-10, mainly produced by Th2 cells and macrophages, can downregulate certain macrophage functions via the inhibition of cytokine production, including TNF-α and IFN-γ, by Th1 cells. IL-10 can also impair macrophage activation by IFN-γ and, therefore, suppress activation of NO-synthase and leishmaniacidal macrophage activity (for a review, see Mosmann and Moore, 1991). Progressive infection by *L. major* in BALB/c mice is accompanied by the expression of IL-10 mRNA in CD4 T cells (Heinzel et al., 1991). It is possible that our mIFN-γ-expressing rBCG stimulates the production of IL-10, particularly late in infection, which could impair macrophage function and result in an exacerbation of infection.

Since the effects of BCG immunization and treatment on leishmanial infections depends on many factors, such as the dose and the route of administration (For a review, see Frommel and Lagrange, 1989), more experiments should be carried out to study the effects of different doses or different routes of immunization, such as intradermally or intraperitoneally, using the mIFN-γ-expressing rBCG.

5. BIBLIOGRAPHY

Behin, R., J. Mauel, and B. Sordat. 1979. *Leishmania tropica* pathogenicity and *in vitro* macrophage function in strains of inbred mice. Exp. Parasitol. 48: 81-91.

Belosevic, M., D.S. Finbloom, M.S. Meltzer, and C.A. Nacy. 1990. IL-2: A cofactor for induction of activated macrophage resistance to infection. J. Immunol. 145: 831-839.

Belosevic, M., D.S. Finbloom, P.H. Van der Meide, M.V. Slayter, and C.A. Nacy. 1989. Administration of monoclonal anti-IFN-gamma antibodies *in vivo* abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. J. Immunol. 143: 166-74.

Bogdan, C., A. Gessner, and M. Rollinghoff. 1993. Cytokines in leishmaniasis: A complex network of stimulatory and inhibitory interactions. Immunobiol. 189: 356-396.

Bogdan, C., H. Moll, W. Solbach, and M. Rollinghoff. 1990. Tumor necrosis factors-alpha in combination with interferon-gamma, but not with interleukin-4 activates murine macrophages for elimination of *Leishmania major*. J. Immunol. 20: 1131-35.

Boom, W.H., L. Liebster, A.K. Abbas, and R.G. Titus. 1990. Patterns of cytokine secretion in murine leishmaniasis: Correlation with disease progression or resolution. Infect. Immun. 58: 3863-70.

Buchmeier, N.A., and R.D. Schreiber. 1985. Requirement of endogenous interferon-γ production for resolution of *Listeria monocytogenes* infection. Proc. Natl. Acad. Sci. USA 82: 7404-7408.

Buchmuller and Mauel. 1979. Studies on the mechanisms of macrophage activation II. Parasite destruction in macrophages activated by supernatants from concanavalin A-stimulated lymphocytes. J. Exp. Med. 150: 359-370.

Connel, N.D., E. Medina-Acosta, W.R. Mcmaster, B.R. Bloom, and D.G. Russell. 1993. Effective immunization against cutaneous leishmaniasis with recombinant Bacille Calmettle-Guérin

expressing the leishmania surface proteinase gp63. Proc. Natl. Acad. Sci. USA. 90: 11473-11477.

Cunha, F.Q., J. Assreuy, S. Moncada, and F.Y. Liew. 1993. Phagocytosis and induction of nitric oxide synthase in murine macrophages. Immunology 79: 408-411.

Flesch, I.E., J.H. Hess, I.P. Oswald, and S.H. Kaufmann. 1994. Growth inhibition of *Mycobacterium bovis* by IFN-gamma stimulated macrophages; regulation by endogenous tumor necrosis factor-alpha and by IL-10. Int. Immunol. 6: 693-700.

Fortier, A.H., B.A. Mock, M.S. Meltzer, and C.A. Nacy. 1987. *Mycobacterium bovis* BCG-induced protection against cutaneous and systemic *Leishmania major* infections of mice. Infect. Immun. 55: 1707-1714.

Gray, P.W., and D.V. Goeddel. 1983. Cloning and expression of murine immune interferon cDNA. Proc. Natl. Acad. Sci. USA 80: 5842-5846.

Green L.C., D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Whisnok, and S.R. Tannenbaum. 1982. Analysis of nitrate, nitrite, and ^{15}N-nitrate in biological fluids. Anal. Biochem. 126: 131-138.

Greenlund, A.C., C.A. Wenner, and R.D. Schreiber 1992. Analysis of IFN-γ receptor binding to IFN-γ. J. Interferon Res. (Abst.) 12: S103.

Handman, E., R. Ceredig, and G.F. Mitchell. 1979. Murine cutaneous leishmanisis: disease patterns in intact and nude mice of various

genotypes and examination of some differences between normal and infected macrophages. Aust. J. Biol. Med. Sci. 57: 9-29.

Heinzel, F.P., M.D. Sadick, B.J. Holaday, R.L. Coffman, and R.M. Locksley. 1989. Reciprocal expression of interferon gamma and IL-4 during the resolution or progression of murine leishmaniasis. evidence for expression of distinct helper T cell subsets. J. Exp. Med. 169: 59-72.

Heinzel, F.P., M.D. Sadick, S.S. Mutta, and R.M. Locksley. 1991. Production of IFN-γ, IL-2, IL-4 and IL-10 by CD4+ lymphocytes *in vivo* during healing and progressive murine leishmaniasis. Proc. Natl. Acad. Sci. USA 88: 7011-15.

Howard, J. G., C. Hale, and F.Y. Liew. 1980. Immunological regulation of experimental cutaneous leishmaniasis III: nature and significance of specific suppression of cell medical immunity in mice highly susceptible to *Leishmania tropica*. J. Exp. Med. 152: 594-607.

Jackson, P.R., M.G. Pappas, and B.D. Hansen. 1985. Fluorogenic substrate detection of viable intracellular and extracellular pathogenic protozoa. Science 227: 435-438.

Kellina, O.I. 1973. Differences of susceptibility of inbred mice of different strains to *Leishmania tropica major*. Med. Parazitol. Parazit. Bolezni 42: 279-285.

King, D.P., and P.P. Jones. 1983. Induction of Ia and H-2 antigens on a macrophage cell line by murine interferon. J. Immunol. 131: 315-318.

Liew, F. Y., Y. Li, and S. Millott. 1990. Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide. J. Immunol. 145: 4306-10.

Liew, F.Y. 1989. Functional heterogeneity of $CD4^+$ T cells in leishmaniasis. Immunology Today 10: 40-45.

Liew, F.Y., S. Millott, Y. Li, R. Lelchuk, W.L. Chan, and H. Ziltener. 1989. Macrophage activation by interferon-γ from host-protective T cells is inhibited by interleukin (IL) 3 and IL-4 produced by disease-promoting T cells in leishmaniasis. Eur. J. Immunol. 19: 1227-1232.

Liew, F.Y., Y. Li, A. Severn, S. Millott, J. Schmidt, M. Salter, and S. Moncada. 1991. A possible novel pathway of regulation by murine T helper type-2 (Th2) cells of a Th1 cell a activity via the modulation of the induction of nitric oxide synthase in the macrophages. Eur. J. Immunol. 21: 2489-2494.

Mauel, J., R. Behin, Biroum-Noerjasin, and D.S. Rowe. 1975. Mechanisms of protective immunity in experimental cutaneous leishmaniasis of the guinea pig. I. Lack of effects of immune lymphocytes and activated macrophages. Clin. Exp. Immunol. 20: 339-350.

Molyneux, D.H., and R.W. Ashford. 1983. Leishmaniasis. In: The Biology of Trypanosoma and Leishmania, Parasites of Man and Domestic Animals. Edited by Molyneux, D.H. and R.W. Ashford. Taylor and Francis Ltd. (London). pp181-249.

Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clone. I definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136: 2348-57.

Mosmann, T.R., and K.W. Moore. 1991. The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol. Today 12: A49-A53.

Mulkerrin, M.G., and R. Wetzel. 1989. pH dependence of the reversible and irreversible thermal denaturation of gamma interferon. Biochemistry 28: 6556-61.

Murry, H.W., H. Masur, and J.S. Keithly. 1982. Cell-mediated immune response in experimental visceral leishmaniasis. I: Correlation between resistance to *Leishmania donovani* and lymphokine-generating capacity. J. Immunol 129: 344-349.

Nacy, C.A., and C.L. Diggs. 1981. Intracellular replication of *Leishmania tropica* in mouse peritoneal macrophages: comparison of amastigote replication in adherent and nonadhanrent macrophages. Infect. Immun. 34: 310-313.

Nacy, C.A., A.H. Fortier, M.G. Pappas, and R.R. Henry. 1983. Susceptibility of inbred to *Leishmania tropica* infection:

correlation of susceptibility with *in vitro* defective macrophage microbicidal activities. Cell. Immunol. 77: 298-307.

Pfizenmaier, K., H. Bartsch, P. Scheurich, B. Seliger, U. Ucer, K, Vehmeyer, and G.A. Nagel. 1985. Differential γ-interferon response of human colon carcinoma cells: Inhibition of proliferation and modulation of immunogenicity as independent effects of γ-interferon on tumor cell growth. Cancer Res. 45: 3503-3509.

Ridley, D.S. 1988. Leishmania and Leishmaniasis. In: Pathogenesis of Leprosy and Related Diseases edited by Ridley, D.S. Butterworth&co. Ltd. pp175-180.

Rubenstein, S., P.C. Familletti, and S. Pestka. 1981. Convenient assay for interferons. J. Virol. 37: 755-758.

Sacks, D.L., J.A. Louis, and D.F. Wirth. 1993. Leishmaniasis. In: Immunology and Molecular Biology of Parasitic diseases. Edited by Warren, K.S. Blackwell Scientific Publications, Boston, PP 237-268.

Sacks, P.L., P.A. Scott, R.F. Asofsky, and A. Sher. 1984. Cutaneous leishmaniasis in anti-IgM-treated mice: enhanced resistance due to functional depletion of a B cell-dependent T cell involved in the suppressor pathway. J. Immunol. 132: 2072-77.

Sadick, M., N. Street, T.R. Mosmann, and R.M. Locksley. 1991. Cytokine regulation of murine leishmaniasis: Il-4 is not sufficient to mediate progressive disease in resistant C57BL/6 mice. FASEB J. 5: A1369 (Abstr.).

Sadick, M.D., R.M. Locksley, C. Tubbs, and H.V. Raff. 1986. Murine cutaneous leishmaniasis: resistance correlates with the capacity to generate interferon-γ in response to leishmania antigens *in vitro*. J. Immunol. 136: 655-660.

Sadick, M.D., F.P. Heinzel, B.J. Holaday, R.T. Pu, R.S. Dawkins, and R.M. Locksley. 1990. Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. J. Exp. Med. 171: 115-127.

Scott, P. 1991. IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. J. Immunol. 147: 3149-55.

Scott, P., P. Natovitz, and A. Sher. 1986. B lymphocytes are required for generation of T cells that mediate healing of cutaneous leishmaniasis. J. Immunol. 137: 1017-21.

Scott, P., P.Natovitz, R.L. Coffman, E. Pearce, and A. Sher. 1988. Immunoregulation of cutaneous leishmaniasis T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. J. Exp. Med. 188: 1675-84.

Sher, A. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu. Rev. Immunol. 10: 385-409.

Stefani, M.M.A., I. Muller, and J. Louis. 1993. *Leishmania major* infection in BALB/c mice: Protection or exacerbation by treatment with different doses of BCG. Res. Immunol. 144: 233-243.

Titus, R.G., A. Kelso, and J.A. Louis. 1984. Intracellular destruction of *Leishmania tropica* by macrophages activated with macrophage activating factors/interferon. Clin. Exp. Immunol. 55: 157-165.

Titus, R.G., R. Ceredig, J.C. Cerottini, and J.A. Louis. 1985. Therapeutic effect of anti-L3T4 monoclonal antibody GK1.5 on cutaneous leishmaniasis in genetically susceptible BALB/c mice. J. Immunol. 135: 2108-2114.

Weintraub, J., and F.I. Weinbaum. 1977. The effect of BCG on experimental cutaneous leishmaniasis in mice. J. Immunol. 118: 2288-2290.

Wilson, M. 1993. Leishmaniasis. Curr. Opin. Infect. Dis. 6: 331-340.

Table 4.1 Primers used for constructing the mIFN-γ expression plasmids

DKU69: CTGaagcttATTGGGACAATCTCT[1]
DKU70: CTAggatccGCACGGCACAGTCATTG[2]
DKU 76: GGTctagacTTTCGCCCGAATCGA[3]
DKU81: ctgtgccgtgCGCGCCCGCGGTTGCCGCTC[4]
DKU82: ccgcgggcgcgCACGGCACAGTCATTGAAAG[5]

Notes:

1. Lower case letters indicate the added HindIII site.
2. Lower case letters indicate the added BamHI site.
3. Lower case letters indicate the added XbaI site.
4. Lower case letters indicate the sequence identical to the mIFN-γ 5' encoding sequence.
5. Lower case letters indicate sequence identical to α-antigen signal peptide 3' sequence.

Table 4.2 Expression of mIFN-γ in BCG

	BCG Colonies (μg DNA)	mIFN-γ (ng)[a] in Supernatants	mIFN-γ (ng)[b] in Cell Sonicates
pMV261-CI(mIFN-γ)	500-1000	6.22 (76%)	2.0 (24%)
pMV261-CII(mIFN-γ)	0		
pMV261-CIII(mIFN-γ)	0		
pMV306-CI(mIFN-γ)	500-1000	ND	ND[c]
pMV306-CII(mIFN-γ)	500-1000	0	0
pMV306-CIII(mIFN-γ)	500-1000	0.5 (14%)	3.0 (86%)

Notes:

a. mIFN-γ detected by ELISA is from 1 ml of a saturated culture of various rBCG. All samples were done in triplicate, and the stardard error of means were all less than 5%.

b. mIFN-γ detected by ELISA is from the sonicates of 1 ml of a saturated culture of various rBCG and 1 ml of a saturated culture of BCG cells generally weighs 10 mg.

c. ND means not done.

Figure 4.1 Plasmids used for expressing mIFN-γ in BCG.

A. Two *E. coli*/BCG shuttle plasmid vectors pMV306 and pMV261 used for making expression plasmids. Explanation for these plasmid vectors can be found in the Results.

B. Three expression cassettes, CI (mIFN-γ), CII (mIFN-γ) and CIII (mIFN-γ), constructed for expressing mIFN-γ. Explanation for making these constructs can be found in the Materials and Methods.

Insertion of three expression cassettes into two vectors generates six expression plasmids, pMV261-CI (mIFN-γ), pMV261-CII (mIFN-γ), pMV261-CIII (mIFN-γ), pMV306-CI (mIFN-γ), pMV306-CII (mIFN-γ), pMV306-CIII (mIFN-γ). All three expression cassettes were inserted into the XbaI and HindIII sites of the vectors.

The restriction enzymes shown in the map are: K, KpnI; P, PstI; E, EcoRI; H, HindIII; B, BamHI; Bg, BlgII; N, NotI; S, SalI; X, XbaI.

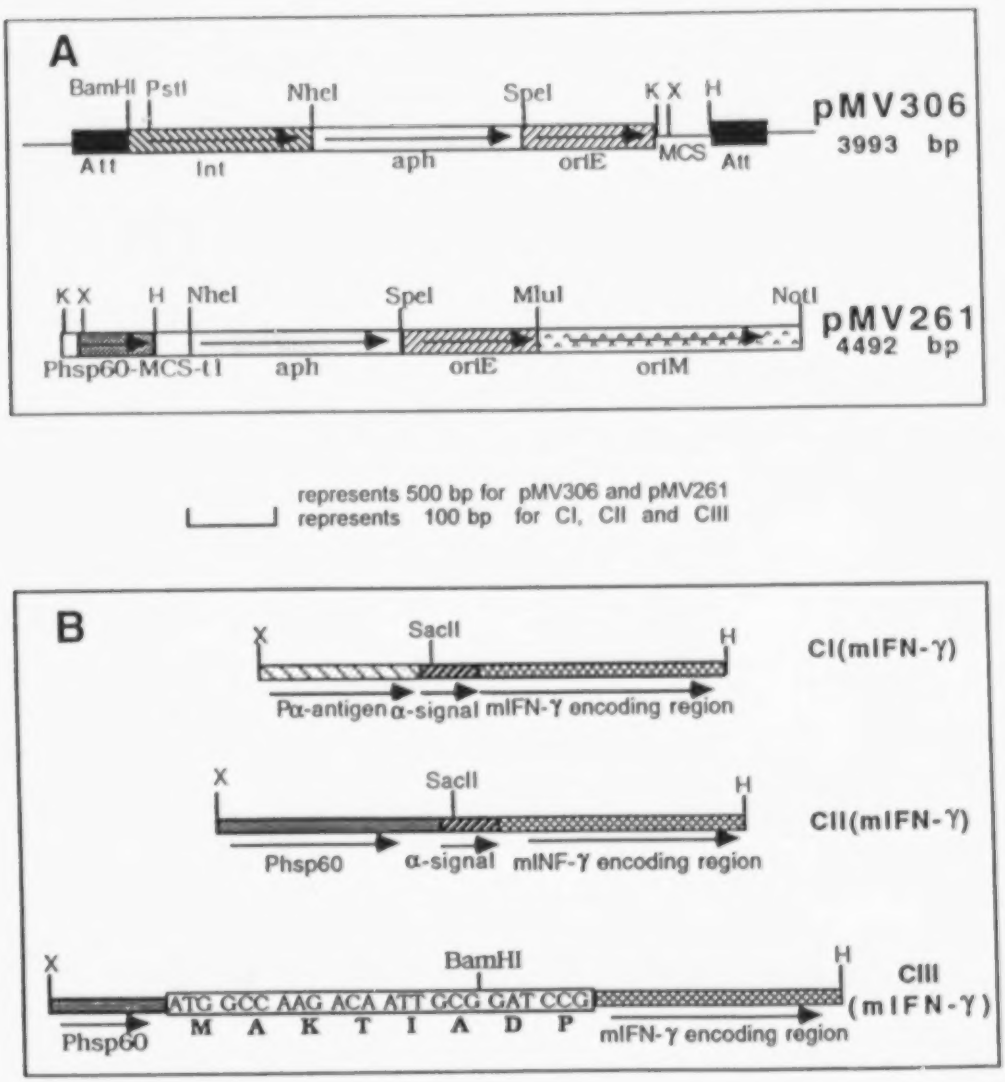
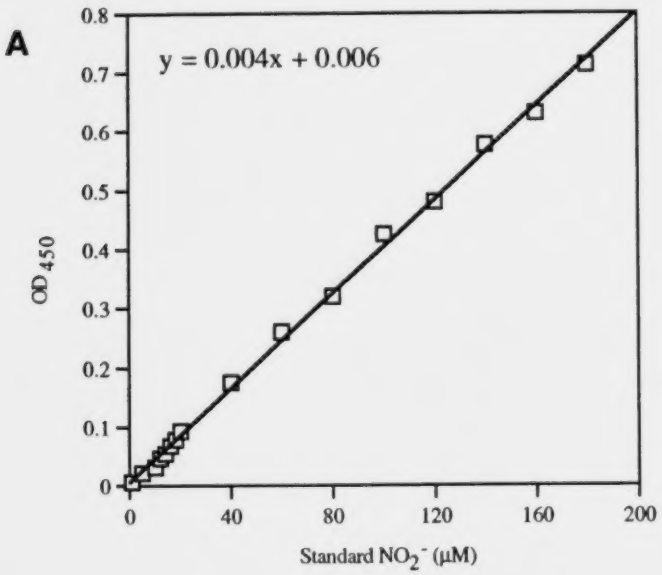

A
BamHI PstI
NheI
SpeI
K X H
pMV306
3993 bp
Att
Int
aph
oriE
MCS
Att
K X H NheI
SpeI
MluI
NotI
pMV261
4492 bp
Phsp60-MCS-tl
aph
oriE
oriM
represents 500 bp for pMV306 and pMV261
represents 100 bp for CI, CII and CIII
B
X
SacII
H
CI(mIFN-γ)
Pα-antigen α-signal mIFN-γ encoding region
X
SacII
H
CII(mIFN-γ)
Phsp60
α-signal
mINF-γ encoding region
X
BamHI
H
CIII
(mIFN-γ)
ATG GCC AAG ACA ATT GCG GAT CCG
M A K T I A D P
Phsp60
mIFN-γ encoding region

Figure 4.2 Production of NO_2^- from J774A.1 cells after stimulation with mIFN-γ prepared from cultures of BCG recombinants in the presence of LPS (20 ng/ml). Measurement of NO_2^- concentration is described in Materials and Methods. All samples were done in triplicate, and the standard error of means were all less than 5%.

A. Standard curve showing the linear relationship between NO_2^- concentration and the OD_{450} from the Griess reaction.

B. NO_2^- (μM) produced from J774A.1 cells after stimulation with the cell sonicates prepared from the cultures of two mIFN-γ-expressing BCG recombinants. 200 u/ml of mIFN-γ (R&D) was used as a positive control. No NO_2^- was produced from J774A.1 cells after stimulation with the supernatants of the two BCG recombinants.


A
y = 0.004x + 0.006
OD 450
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
40
80
120
160
200
Standard NO2- (µM)

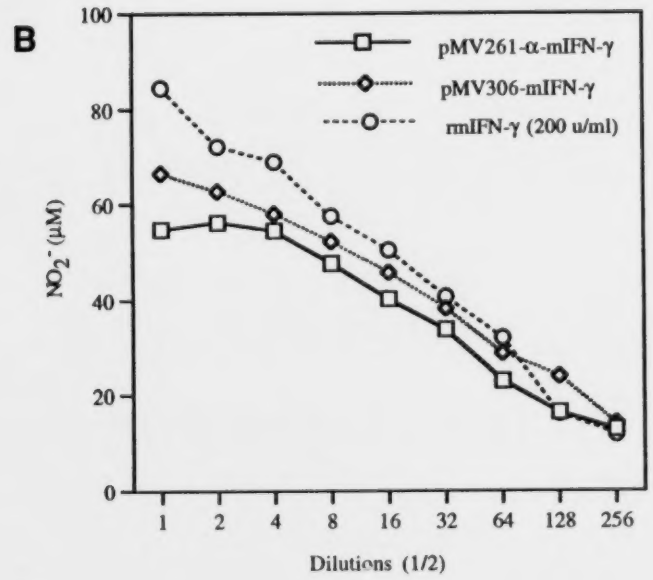

B
pMV261-α-mIFN-γ
pMV306-mIFN-γ
rmIFN-γ (200 u/ml)
NO2- (µM)
100
80
60
40
20
0
1
2
4
8
16
32
64
128
256
Dilutions (1/2)

Figure 4.3 Stability test of mIFN-γ in Dubos medium in the BCG growth condition. 100 ml Dubos-ADC medium containing 200 u/ml mIFN-γ (R&D Systems) was grown in a 1000 ml roller bottle at 37°C for two weeks. At different time intervals, 1 ml of medium was removed from the culture and stored at -80°C. The activity of mIFN-γ remaining in the medium was assayed as described in Materials and Methods. All samples were done in triplicate, and the standard error of means were all less than 5%.

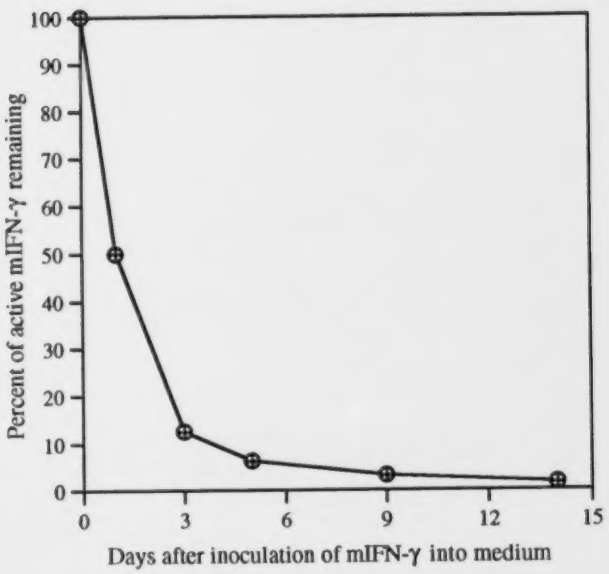
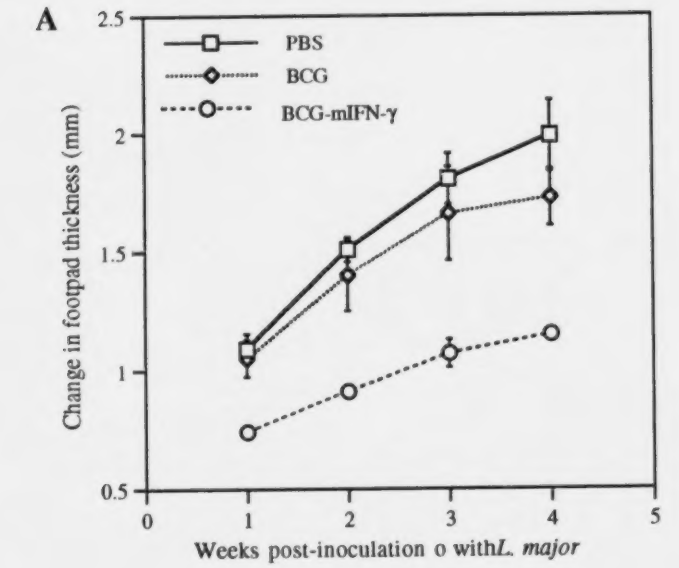

Percent of active mIFN-γ remaining
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
Days after inoculation of mIFN-γ into medium

Figure 4.4 Development of cutaneous disease in BALB/c mice infected with 10^6 amastigotes of *L. major* two weeks after intraperitoneal immunization with 10^6 of one of the mIFN-γ-expressing BCG (BCG-pMV261-mIFN-γ, hereafter called BCG-mIFN-γ). BCG and PBS were used as controls. Each point represents the mean ± SEM from 5 mice, and the results shown in this figure represent one of two similar experiments.

A. Change in footpad thickness of the infected mice. Footpad thickness was measured using a dial thickness gauge every week for four weeks, and the change in footpad thichness was determined by subtracting the thickness of the uninfected right footpad from that of the infected left footpad.

B. *L. major* parasite load per infected footpad. The number of parasites in the footpad were determined four weeks after *L. major* infection as described in Materials and Methods.


A
2.5
2
1.5
1
0.5
PBS
BCG
BCG-mIFN-γ
Change in footpad thickness (mm)
0
1
2
3
4
5
Weeks post-inoculation o withL. major

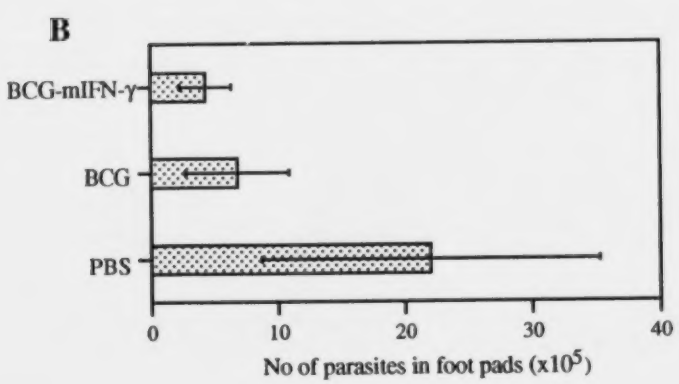

B
BCG-mIFN-γ
BCG
PBS
0
10
20
30
40
No of parasites in foot pads (x10^5)

Figure 4.5 Development of cutaneous disease in BALB/c mice infected with 10^6 amastigotes of *L. major* one week after intraperitoneal immunization with 10^6 of BCG-mIFN-γ. BCG and PBS were used as controls. Each point represents the mean ± SEM from 5 mice, and the results shown in this figure represent one of two similar experiments.

A. Change in footpad thickness of the infected mice. Footpad thickness was measured using a dial thickness gauge every week for four weeks, and the change in footpad thichness was determined by subtracting the thickness of the uninfected right footpad from that of the infected left footpad.

B. *L. major* parasite load per infected footpad. The number of parasites in the footpad were determined four weeks after *L. major* infection as described in Materials and Methods.

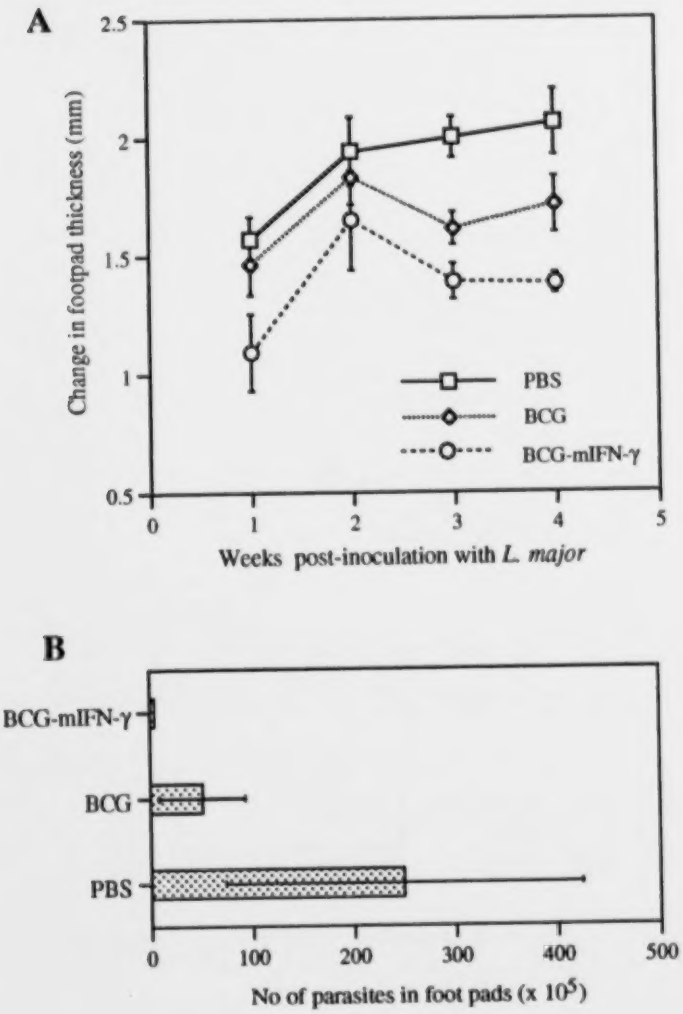
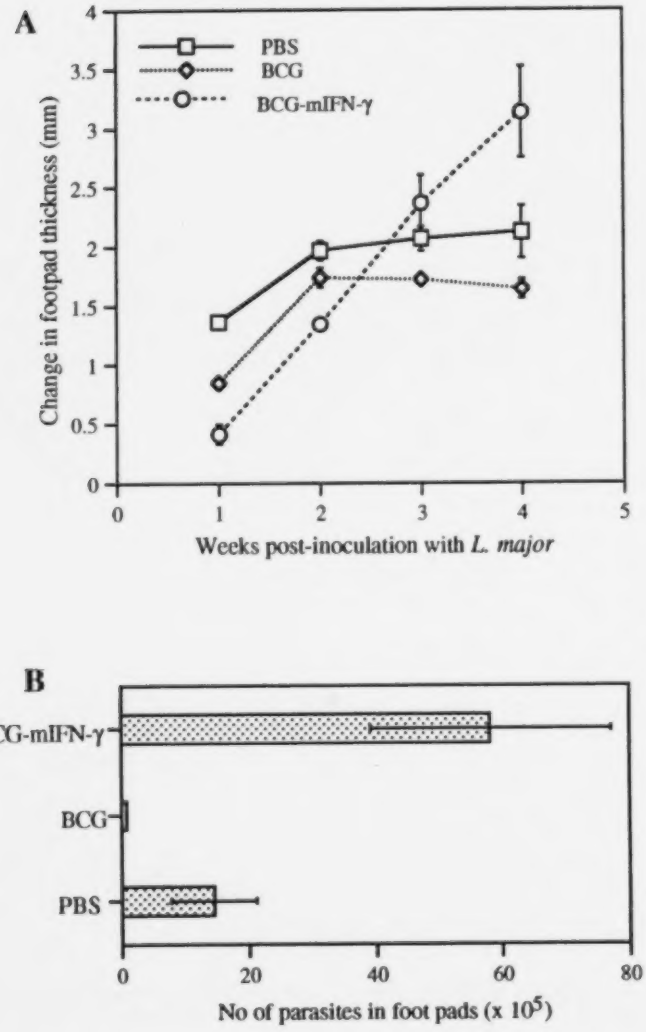

A
2.5
2
1.5
1
0.5
Change in footpad thickness (mm)
PBS
BCG
BCG-mIFN-γ
0
1
2
3
4
5
Weeks post-inoculation with L. major
B
BCG-mIFN-γ
BCG
PBS
0
100
200
300
400
500
No of parasites in foot pads (x 10^5)

Figure 4.6 Development of cutaneous disease in BALB/c mice infected with 10^6 amastigotes of *L. major* three weeks after intraperitoneal immunization with 10^6 of BCG-mIFN-γ. BCG and PBS were used as controls. Each point represents the mean ± SEM from 10 mice, and the results shown in this figure represent one of two similar experiments.

A. Change in footpad thickness of the infected mice. Footpad thickness was measured using a dial thickness gauge every week for four weeks, and the change in footpad thickness was determined by subtracting the thickness of the uninfected right footpad from that of the infected left footpad.

B. *L. major* parasite load per infected footpad. The number of parasites in the footpad were determined four weeks after *L. major* infection as described in Materials and Methods.


A
PBS
BCG
BCG-mIFN-γ
Change in footpad thickness (mm)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
1
2
3
4
5
Weeks post-inoculation with L. major
B
BCG-mIFN-γ
BCG
PBS
0
20
40
60
80
No of parasites in foot pads (x 10^5)

Chapter V

Mutagenesis of Murine Interleukin 5 for Crystal Analysis

This work has been presented at the Experimental Biology 94, California, USA and published in abstract form. **Kong, D.**, Boodhoo, A., Read, D. and Kunimote, D. 1994. Mutagenesis of murine IL-5 for crystal analysis. *The FASTB Journal* 8: A510.

The preliminary X-ray analysis of mIL-5 structure has been published. Boodhoo, A., Duke, N., **Kong, D.**, Ritzel, D., Kunimoto, D. and Read, R. 1994. Purification, crystallization and preliminary X-ray analysis of murine interleukin 5. *J. of Molecular Biology* 241: 269-272.

The spatial structure of mIL-5 is being prepared for publication. Sharp, A. M., Boodhoo, A., Duke, N.E., **Kong, D.**, Kunimoto, D.Y. and Read, R.J. 1995. Molecular replacement solution of murine interleukin 5 (In preparation).

* mIL-5 protein was purified by A. Boodhoo and the isoelectric focusing gel present in this chapter was also performed by A. Boodhoo.

1. INTRODUCTION

Murine interleukin 5 (mIL-5), originally named B cell growth factor (BCGF-II), T cell replacing factor (TRF) and eosinophil differentiating factor (EDF), is a multi-functional T cell produced cytokine, that can stimulate B cell growth and differentiation, enhance T cell cytotoxity and induce eosinophil differentiation (reviewed in: McKenzie and Sanderson, 1992; Takatsu *et al.*, 1994)). As a B cell growth factor, mIL-5 can induce the proliferation of dextran sulfate-stimulated normal B cells and the mouse B cell lymphoma BCL1 (Howard *et al.*, 1982; Swain *et al*, 1983). As a B cell differentiation factor, mIL-5 can induce the terminal differentiation of B cells to Ig-secreting cells (Takatsu *et al.*, 1985; Swain, 1985; Pene *et al.*, 1989). The studies on the effects of human IL-5 on the growth and differentiation of B cells produced conflicting results; while some laboratories found no activity (Clutterbuck *et al.*, 1987), others described activity similar to the murine system (Azuma *et al.*, 1983). As an eosinophil differentiation factor, IL-5 (from both human and mouse) has the ability to stimulate bone marrow cells to differentiate into eosinophils (Sanderson *et al.*, 1986; Begley *et al.*, 1986; Yamaguchi *et al.*, 1988). In addition, both human and mouse IL-5 have chemotactic activity for eosinophils and probably have a role in degranulation of eosinophils (Lopez *et al.*, 1988; Fujisawa *et al.*, 1990). Furthermore, mIL-5 can enhance the activity of IL-2 to induce cytotoxic T cells and therefore, has been given another name, "killer helper factor" (Takatsu *et al.*, 1987; Ramos, 1989).

Cloned mIL-5 cDNA encodes a mature polypeptide of 113 amino acids with a predicted molecular weight of 13 KDa (Kinashi *et al.*, 1986). Native mIL-5 is a disulfide-linked homodimer and the dimerization is essential for its biological activities (Takatsu *et al.*, 1988; Tsuruoka *et al.*, 1990).

mIL-5 is a glycoprotein with three potential asparagine glycosylation sites, two of which are in hydrophilic regions and thus potentially N-glycosylated (Bond *et al.*, 1987). Glycosylation has been shown to be unnecessary for biological activity since removal of the carbohydrates by deglycosylation enzyme did not reduce the function of mIL-5 (Kunimoto *et al.*, 1991; Tominaga *et al.*, 1990). The natural mIL-5 and recombinant mIL-5 expressed by eukaryotic expression systems display several bands on SDS-PAGE or isoelectric focusing gels with the molecular weights in the range of 45-60 KDa due to the heterogeneous glycosylation of mIL-5 (Kunimoto *et al.*, 1991; Kinasni *et al.*, 1986).

While the crystal structure of huIL-5 has been solved (Milurn *et al.*, 1993), the structure of mIL-5 remains unknown. To study the mIL-5 three-dimensional structure, milligram quantities of functional mIL-5 were produced by using the baculovirus expression system (Kunimoto *et al.*, 1991). Initial attempts to crystallize recombinant mIL-5 resulted in low resolution diffracting crystals, this was presumably due to the heterogeneous glycosylation. To make mIL-5 more homogenous, we undertook to mutate 2 potential N-glycosylation sites.

In this chapter, I report the *in vitro* mutagenesis of mIL-5, expression and purification of wild-type and mutated mIL-5 for crystal analysis of the mIL-5 structure.

2. MATERIALS AND METHODS

Materials

Baculovius expression vector pVL1393, *Spodoptera frugiperda* (Sf9) cells and *Autographa californica* multiply-enveloped nuclear polyhedrosis (AcMNPV) wild type viral DNA are all M. D. Summers (Texas Agricultural Experimental Stations)

Grace's Antheraea medium (GIBCO-BRL, Gaithersburg, MD) was used for short term incubations of Sf9 cells, such as seeding cells, transfection etc. TNM-FH medium, prepared from Grace's medium by the addition of 3.3 g/liter Yeastolate and 3.3 g/liter Lactalbumin Hydrolysate (Difco Laboratories, Detroit, MI), was used for routine growth of Sf9 cells in suspension. Complete growth medium, made from TNM-FH by the addition to the final concentration of 10% fetal bovine serum (FBS) and 25 μg/ml gentamicin, was used for the maintenance of Sf9 cells.

E. coli DH5α and vectors pBluescriptKS(+) and pGEM were from Stratagene Cloning Systems (La Jolla, Calif.).

The oligonucleotide primers used for PCR were synthesized as described in previous chapters and shown below.

T3: ATTAACCCTCACTAAAG

T7: AATACGACTCACTATAG

DKU9 (pVL5'): GGATTATTCATACCGTCC

DKU11 (pVL3'): TCCAACGACAAGCTTCATCG

DKU28: GACAAGCcAgGAGACGATGAG

DKU57: GAAGcAgCAAACTGTCCGTG

(Lower case letters in DKU28 and DKU57 indicate the nucleotide sequences used to replace Asn with Gln)

Cloning of mIL-5 cDNA to pBluescriptKS(+)

mIL-5 cDNA was originally cloned in a pCM55 vector, which was kindly provided by Dr. John Elliott and Dr. Vern Paetkau, who cloned it from a EL-4 T cell line (the sequence was published in Kinashi *et al.*, 1986). To facilitate the *in vitro* mutagenesis with PCR and later cloning, an EcoRI-AccI fragment of mIL-5 cDNA, in which most of untranslated region was removed, was cloned into pGEM-3 vector and then into the EcoRI and PstI sites of the multiple cloning sites (MCS) of pBluescript KS(+) as shown in Figure 5.1.

***In vitro* Mutagenesis of mIL-5**

Mutagenesis of mIL-5 was carried out by a modification of the method described by Landt *et al.* (1990) and Kuipers *et al.* (1991) with two rounds of PCR and shown in Figure 5.2. The first round PCR was carried out in a total volume of 100 µl with 1 ng template DNA, 1.5 µM of each primer, 100 µM of each dNTP, 1 units of Taq DNA polymerase in reaction buffer containing 10 mM Tris-HCl (pH, 9.0), 1.5 mM MgCl2, 50 mM KCl. 0.1% Triton X-100 and 0.01% w/V gelatin. The reaction mixture was overlaid with 100 µl light mineral oil, boiled for 3 minutes, and then amplified in a PHC-2 thermocycler (Techne Inc., Princeton, New Jersey) for 30 cycles consisting of 1 minute at 94 C, 1 minute at 55 C and 1 minute at 72 C. The second

round PCR was done using the same conditions as the first round PCR except that the DNA fragment isolated from the first round PCR was used as one of the two primers. The DNA fragment amplified by PCR was electrophoreses in a 1% agarose gel and purified with a Gene Clean kit (BIO/CAN, Mississauga, ON) according to the procedures provided by the supplier. After PCR mutagenesis, the mutated mIL-5 sequence was confirmed by DNA sequencing and then cloned into the EcoRI and PstI sites of the baculovirus expression vector pVL1393

Expression of mIL-5 proteins

Wild type and mutant forms of mIL-5 were expressed in the baculovirus expression system according to Summers and Smith (1987).

a). Transfection of Sf9 cells

Spodoptera frugiperda (sf9) cells were coinfected with pVL1393-mIL-5 (wild type and mutants) and wild type baculovirus DNA. 2 x 10^6 Sf9 cells were seeded into 25 cm^2 flasks. After one hour incubation at room temperature, the medium was removed from flasks and 0.75 ml of Grace's medium with 10% FBS and 25 µg/ml of gentamicin was added to each flasks. 1 µg of AcMNPV wild type viral DNA and 2 µg of pVL1393-mIL-5 plasmid DNA were mixed in a 1.5 ml polypropylene tube containing 0.75 ml of transfection buffer (25 mM HEPES, pH 7.1, 140 mM NaCl, 125 mM Cacl2). The mixture of the DNA-transfection buffer was added dropwise to a flask containing seededSf9 cells. The flask was incubated at room temperature for 4 hours and then the medium

from each flask was removed and replaced with 5 ml of complete medium. The flask was incubated at room temperature in a humid environment for 4-6 days to visualize the signs of infection, which include the appearance of polyhedra, increase in the diameter of cells and cell lysis later in infection.

b). Visual screening for recombinant viruses

Recombinant viruses were purified by 3 rounds of visual selection of occlusion-negative plaques.

2 x 10^6 Sf6 cells were seeded into 60 x 15 mm culture plates. After one hour incubation, the medium was removed and 1 ml of 10^{-3} through 10^{-5} dilutions of the transfection mixtures were inoculated into each plate. The plate was incubated for one hour at room temperature, and then 4 ml of1.5% low melting point (Sea Plaque) agarose dissolved in Grace's medium with10% FBS were overlaid onto each plate. After the agarose solidified, the plates were incubated in a humid environment until the plaques were well formed (generally 4-6 days). The plates were examined under a dissecting microscope. The wild-type plaques (occlusion-positive) look very refractile, almost crystal-like, with a slight yellow while the recombinant plaques (occlusion-negative) plaques are less refractile and light gray instead of light yellow. The recombinant viral plaques were removed using a 200 µl pipette tips and subjected to another round screening. To obtain a pure recombinant virus stock, three round visual screening was carried out. The presence of recombinant virus was confirmed with PCR in every round of screening by using 2 primers DKU9 (pVL 5')and DKU11 (pVL 3').

c). Infection of Sf9 cells with the recombinant viruses

Infection of Sf9 cells with the recombinant viruses were described in Kunimoto *et al.* (1991). Briefly, Sf9 cells were infected with various recombinant baculoviruses with wild-type or mutated mIL-5 at a multiplicity of infection of 10. The infected cells were adjusted to 2 x 10^6/ml in Grace's medium, and then grown in suspension for three days. The supernatant was used for preparation of mIL-5 protein.

Purification of mIL-5 for crystallization

Two liter of supernatants from recombinant mIL-5-baculovirus infected sf9 cell cultures were concentrated on an Amicon YM5 filter ten fold. This concentrated solution was passed through a DEAE-sephacel column equilibrated and eluted with 25 mM Tris-HCl buffer (pH 8). The eluted solution was further concentrated to 10 ml and then was applied to a HiLoad 16/10 Q sepharose HP column (Pharmacia-LKB) equilibrated with 20 mM ethanolamine buffer (pH 9.2) and eluted with a NaCl gradient (0 to 0.5 M) in the same buffer. mIL-5 proteins in the different fractions, monitored by bioassay and SDS-PAGE gel, were poured together, concentrated and then dialyzed in a solution (5 mM Tris-HCl and 0.02% Sodium azide). The purified mIL-5 by these procedures was used for crystallization experiments.

6. mIL-5 bioassay

TF-1 cells (2 X 10^4/well) were cultured in RIPM 1640 medium with dilutions of mIL-5 samples in a 96-well flat-bottom plate. All

samples were assayed in triplicate. After 28 hr culture at 37 C in 5% CO_2 atmosphere, 20 µL MTT (5 mg/ml in PBS) was added to each well and the cultures were incubated further for 2-4 hours. The color was extracted with 0.04 N HCl in isopropanal, and then read at 540 nm using a Titertek Multiskan MC Apparatus.

3. RESULTS AND DISCUSSION

1. *In vitro* mutagenesis of mIL-5

mIL-5 protein has three potential asparagine glycosylation sites in amino acids 28, 57 and 71 (the numbering system is based on the human IL-5 sequence, see McKenzie and Sanderson, 1992), two of which (Asn28 and Asn57) are in hydrophilic region and thus potentially N-glycosylated. We undertook to mutate the two potential-glycosylation sites. At each site, an asparagine was changed to a structural and charge similar amino acid, glutamine.

To facilitate the mutagenesis with PCR, the mIL-5 encoding sequence was cloned into the MCS of pBluescript SK (+) as described in Materials and Methods and shown in Figure 5.1. The mutagenesis was carried out using two rounds of PCR as shown in Figure 5.2. To mutate Asn28, the first round PCR was done using primers T7 and DKU28, while the primers DKU57 and T7 was used in the first round PCR to mutate Asn57. The second round PCR was carried out using primer T3 and the DNA fragments purified from the first round PCR. After the second round of PCR, the mutated DNA fragments were cloned into pBluescript SK(+) for DNA sequencing. Generally four clones were picked from the cloning of each site. Sequencing of these

clones indicated that all clones had the desired mutations. Mutagenesis using the PCR described in (Landt *et al.*, 1990) provide a very simple and reliable way to generate substitution mutations.

2. Biological activity of mutated mIL-5

The mutated mIL-5 as well as wild-type mIL-5 were expressed in a baculovirus expression system as described in Materials and Methods. Both principles and detailed protocols of this system can be found in Summers and Smith (1987). It has been indicated by some evidence that the glycosylation of mIL-5 protein is not necessary for its biological activity. We confirmed this observation by showing that one of the mutated mIL-5 (Asn57) expressed in baculovirus retained the same biological activity as non-mutated protein at least in a TF-1 cell bioassay (Figure 5.3).

3. Homogeneity of mutated mIL-5 protein

Because of the heterogeneous glycosylation of mIL-5 protein, the recombinant mIL-5 expressed in baculovirus was heterogeneous and displayed several discrete bands with molecular weights in the range of 45-60 KDa. (Kunimoto *et al.*, 1991). The mutated mIL-5 proteins expressed in baculovirus were more homogenous than the non-mutated mIL-5 proteins in an isoelectric focusing gel (data not shown).

From one of the mutated mIL-5 (Asn57) protein, two different crystal forms were obtained. One of the crystal forms, the orthorhombic crystals, diffract to beyond 2.5 A resolution; the second crystal form, belonging to a trigonal space group, diffracts to about

3.8 A resolution (Boodhoo *et al.*, 1994). The study of the mIL-5 three-dimensional structure is in progress.

4. BIBLIOGRAPHY

Azuma, C., T. Tanabe, M. Kunishi, T. Kinashi, T. Noma, F. Matsuda, Y. Yaoita, K. Takatsu, L. Hammarstrom, C.I.E. Smith, E. Severinson, and T. Honjo. 1986. Cloning of cDNA for human T-cell replacing factor (interleukin 5) and comparison with the murine homologue. Nucl. Acids Res. 14: 9149-9158.

Begley, C.G., A.F. Lopez, N.A. Nicola, D.J. Warren, M.A. Vadas, C.J. Sanderson, and D. Metcalf. 1986. Purified colony-stimulating factors enhance the survival of human neutrophils and eosinophils in *vitro*: a rapid and sensitive microassay for colony-stimulating factors. Blood. 68: 162-166.

Bond, M.N., B. Shrader, T.R. Mosmann, and R.L. Coffman. 1987. A mouse T cell product that preferentially enhances IgA production II: Physicochemical characterization. J. Immunol. 139: 3691-3696.

Boodhoo, A., N. Duke, D. Kong, M. Ritzel, D.Y. Kunimoto, and R. Read. 1994. Purification, crystallization and preliminary X-ray analysis of murine interleukin 5. J. Mol. Biol. 241: 269-272.

Clutterbuck, E., J.G. Shield, J. Gordon, S.H. Smith, A. Boyd, R.E. Callard, H.D. Campbell, I.G. Young, and C.J. Sanderson. 1987. Recombinant human interleukin 5 is an eosinophil differentiation factor but has no activity in standard human B cell growth factor assays. Eur. J. Immunol. 17: 1743-1750.

Fujisawa, T., R. Abu-Ghazaleh, H. Kita, C.J. Sanderson, and G.J. Gleich. 1990. Regulatory effect of cytokines on eosinophil degranuation. J. Immunol. 144: 642-646.

Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W.E. Paul. 1982. Identification of a T cell-derived B cell growth factor distinct from interleukin 2. J. Exp. Med. 155: 914-923.

Kinashi, T., N. Harada, E. Severinson, T. Tanabe, P. Sideras, M. Konishi, C. Azuma, A. Tominaga, S. Bergatedt-Lindqvist, M. Takahashi, F. Matsuda, Y. Yaoita, K. Takaysu, and T. Honjo. 1986. Cloning of a complementary DNA encoding T cell replacing factor and identity with B cell growth factor II. Nature (London) 324: 70-73.

Kong, D., Boodhoo, A., Read, D. and Kunimote, D. 1994. Mutagenesis of murine IL-5 for crystal analysis. *The FASEB Journal* 8: A510.

Kuipers, O.P., H.J. Boot, and W.M. Devos. 1991. Improved site-directed mutagenesis method using PCR. Nucleic Acids Res. 19: 4558-4559.

Kunimoto, D.Y., K.C. Allison, C. Watson, T. Fuerst, G. Armstrong, W. Paul, and W. Strober. 1991. High-level production of murine interleukin 5 (IL-5) utilizing recombinant baculovirus expression. Purification of the rIL-5 and its use in assessing the biological role of IL-5 glycosylation. Cytokine 3, 224-230.

Landt, O., H.P. Grunert, and V. Hahn. 1990. A general method for rapid site-directed mutagenesis using the polymerase chain reaction. Gene 96, 125-128.

Lopez, A.F., C.J. Sanderson, J.R. Gamble, H.D. Campbell, I.G. Young, and M.A. Vadas. 1988. Recombinant human interleukin 5 is a selective activator of human eosinophil function. J. Exp. Med. 167: 219-224.

McKenzie, A.N.J., and C.J. Sanderson. 1992. Interleukin-5. In: Interleukins: Molecular Biology and Immunology. Edited by Kishimoto, T. Chem. Immunol. Basel. Karger. Vol.51: 135-152.

Milburn, M.V., A.M. Hassell, M.H. Lambert, S.R. Jordan, A.E.I. Proudfoot, P. Graber, and T.N.C. Wells. 1993. A novel dimer configuration revealed by the crystal structure at 2.4 A resolution of human interleukin 5. Nature (London) 363: 172-176.

Pene. J., I. Chretien, F. Rousset, F. Briere, J.Y. Bonnefoy, and J.E. De Vries. 1989. Modulation of IL-4-induced human IgE production *in vitro* by IFN-gamma and IL-5: the role of soluble CD23 (s-CD23). J. Cell. Biochem. 39: 253-264.

Ramos, T. 1989. Interleukin 5 is a differentiation factor for cytotoxic T lymphocytes. Immunol. Lett. 21: 277-284.

Sanderson, C.J., A. O'Garra, D.J. Warren, and G.G.B. Klaus. 1986. Eosinophil differentiation factor also has B-cell growth factor activity: proposed name interleukin 5. Proc. Nat. Acad. Sci. USA. 83: 437-440.

Summers, M.D., and G.E. Smith. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. College Station, Texas Agricultural Experimental Stations.

Swain, S.L. 1985. Role of BCGF-II in the differentiation to antibody secretion of normal and tumor B cells. J. Immunol. 134: 3934-3943.

Swain, S.L., M. Howard, J. Kappler, P. Marrack, J. Watson, R. Booth, G.D. Wetzel, and R.W. Dutton. 1983. Evidence for two distinct classes of murine B cell growth factor with activities in different functional assays. J. Exp. Med. 158: 822-835.

Takatsu, K., A. Tominaga, N. Harada, S. Mita, M. Matsumoto, T. Takahashi, Y. Kikuchi, and N. Yamaguichi. 1988. T cell-replacing factor (TRF)/interleukin 5 (IL-5): Molecular and functional properties. Immunol Rev. 102: 107-135.

Takatsu, K., N. Harada, Y. Hara, Y. Takahama, G. Yamada, K. Dobashi, and T. Hamaoka. 1985. Purification and physicochemical characterization of murine T cell replacing factor (TRF). J. Immunol. 134: 382-389.

Takatsu, K., S. Takaki, and Y. Hitoshi. 1994. Interleukin-5 and its receptor system: implications in the immune system and inflammation. Adv. Immunol. 57: 145-190.

Takatsu, K., Y. Kikuchi, T. Takahashi, T. Honjo, M. Matsumoto, N. Harada, N. Yamaguchi, and A. Tominaga. 1987. Interleukin 5, a T cell- derived B cell differentiation factor, also induces cytotoxic T lymphocytes. Proc. Natl. Acad. Aci. USA. 84: 4234-4238.

Tominaga, A., T. Takahashi, Y. Kikuchi, S. Mita, S. Noami, N. Harada, N. Yamaguchi, and K. Takaysu. 1990. Role of carbohydrate moiety of

Il-5: Effect of tunicamycin on the glycosyation of IL-5 and the biological activity of deglycosylated IL-5. J. Immunol. 144: 1345-1352.

Tsuruoka, N., K. Funakoshi, S. Fodam, and M. Tsujimoto. 1990. Interaction of interleukin 5 with its receptors on murine leukemia BCL1 cells and its implication in biological activity. Cell. Immunol. 125: 354-362.

Yamaguchi, Y., Y. Hayashi, Y. Sugama, Y. Miura, T. Kasahara, S. Kitamura, M. Torisu, S. Mita, A. Tominaga, and K. Takatsu. 1988. Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs *in vitro* survival: IL-5 as an eosinophil chemotactic factor. J. Exp. Med. 167: 1737-1742.

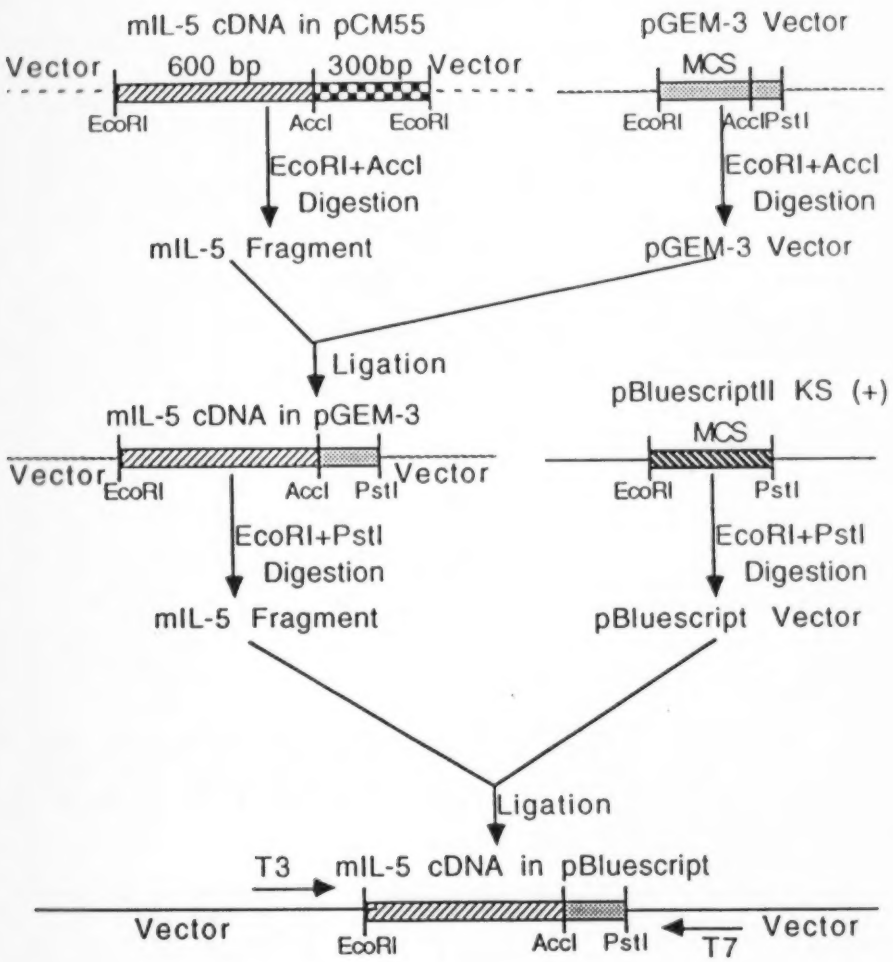


Figure 5.1 Cloning of mIL-5 cDNA from pCM55 to pBluescript SK. The procedures are explained in Material and Methods.

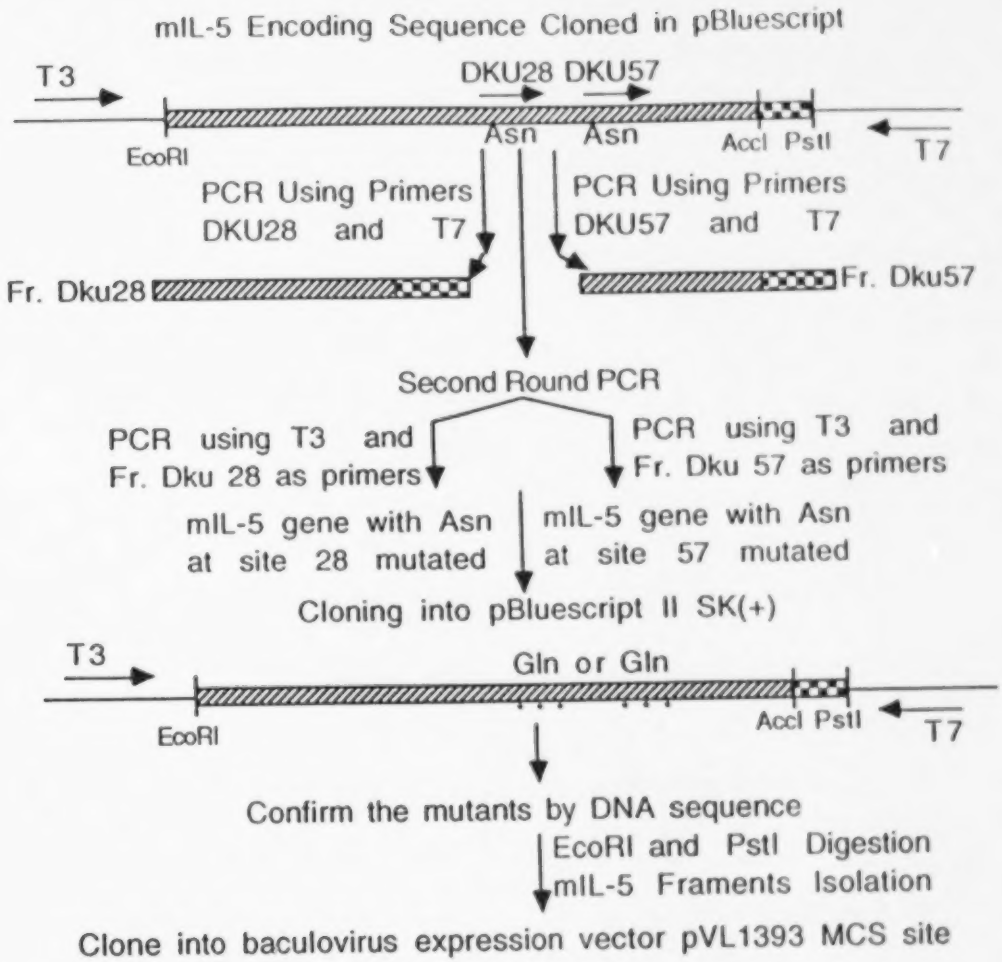


Figure 5.2 *In vitro* mutagenesis of two glycosylation sites of mIL-5 by DNA polymerase chain reaction (PCR) as described in Materials and Methods.

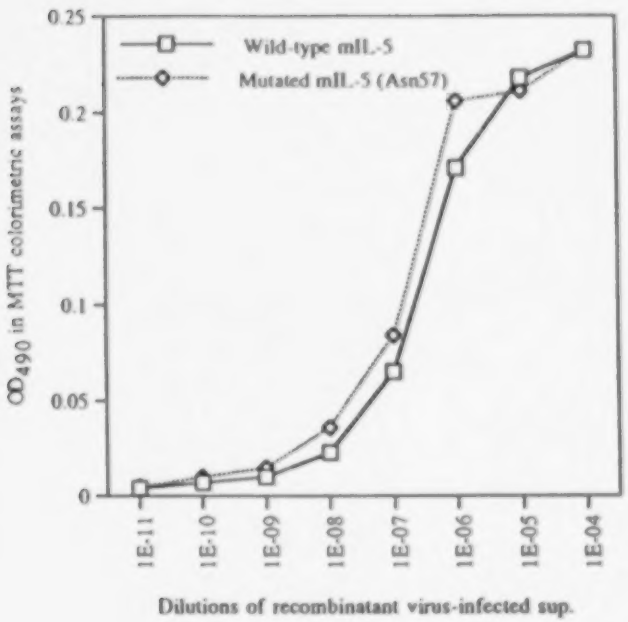


Figure 5.3 TF-1 cell proliferation in response to increasing amounts of recombinant mIL-5, showing that one of the glycosylation-mutated mIL-5 (used for crystal analysis) have the same activity as the wild type mIL-5 in the supernatant of recombinant baculovirus-infected sf9 cell culture. The assay was carried out as described in Materials and Methods.

Chapter VI

General Discussion and Conclusions

1. Summary

During my Ph.D. studies, several BCG recombinants expressing human interleukin 2 (huIL-2), human tumor necrosis alpha (huTNF-α) or mouse interferon gamma (mIFN-γ) were constructed with the use of various techniques of genetic engineering.

The expression of huIL-2 in BCG was reported in Chapter II. Two huIL-2-secreting BCG recombinants, BCG-pMV306-CI (huIL-2) and BCG-pMV306-CII (huIL-2) were constructed. In both BCG recombinants, the expression plasmids were integrated into the chromosome of the BCG Pasteur strain and therefore, stably maintained. BCG-pMV306-CII (huIL-2), in which the huIL-2 encoding sequence was under the control of the promoter of BCG heat shock protein 60 (hsp60), secretes approximately 150 units/ml of huIL-2; BCG-pMV306-CI (huIL-2), in which the huIL-2 encoding sequence was regulated by the promoter of BCG α-antigen, secreted approximately 1/6 the amount of huIL-2 expressed by BCG-pMV306-CII (huIL-2). Administration of these huIL-2-secreting BCG recombinants into BALB/c mice can limit the growth of BCG *in vivo*. Evidence presented in chapter II indicates that the inhibition of BCG growth in BALB/c mice is likely due to the augmentation of the host immune response by secreted huIL-2.

In Chapter III, I reported the expression of huTNF-α in BCG. Two huTNF-α-expressing BCG recombinants, BCG-pMV261-CIII (huTNF-α) and BCG-pMV261-CIII (huTNF-α), were obtained. In both BCG recombinants, the huTNF-α encoding sequence was ligated to the

hsp60 expression cassette using the BamHI; therefore, a fusion huTNF-α protein with 8 amino acids derived from hsp60 protein and the BamHI site was expressed intracellularly in both BCG recombinants. Attempts to construct BCG recombinants secreting biologically active huTNF-α were unsuccessful. BCG-pMV261-CIII (huTNF-α), in which the expression cassette was cloned in the extrachromosomal plasmid pMV261, expressed approximately 2.5 μg/10 mg BCG cells; while BCG-pMV306-CIII (huTNF-α), in which the expression cassette was cloned into the integrative vector pMV306, expressed approximate 40 ng/10 mg of BCG cells. During the expression of huTNF-α in BCG, I felt that huTNF-α could be used as a reasonable reporter gene to study gene expression and regulation in BCG, since no other reporter gene has been available for this purpose. The bioassay of huTNF-α, based on its cytolytic effects on a mouse tumor cell line L-929-8, is simple to perform and highly sensitive. Using huTNF-α as a reporter gene, I studied the effects of initiation codon change on gene expression in BCG. I found that the expression of huTNF-α fused to BCG hsp60 regulatory sequences was increased at least 100 percent when the AUG initiation codon of the hsp60 gene was changed to GUG. This increased expression is accompanied by an increase in huTNF-α specific mRNA. This is the first example showing that GUG is used as a more efficient initiation codon than AUG.

In chapter IV, I reported the expression of mIFN-γ in BCG. I chose to express mouse IFN-γ instead of human IFN-γ because IFN-γ, unlike IL-2 and TNF-α, is highly species-specific and I wanted to use

mice to study various effects of BCG recombinants *in vivo*. Two mIFN-γ-expressing BCG recombinants, BCG-pMV261-CI (mIFN-γ) and BCG-pMV306-CIII (mIFN-γ), were obtained. BCG-pMV261-CI (mIFN-γ), in which the mIFN-γ encoding sequence was directly ligated to the signal peptide and promoter sequence of the α-antigen, expressed approximate 6.22 ng/ml mIFN-γ extracellularly and 2.0 ng/ml intracellularly. On the other hand, most of the mIFN-γ (80%) expressed by another BCG recombinant, BCG-pMV306-CIII (mIFN-γ), was detected intracellularly. No biological activity of mIFN-γ could be detected in the supernatant of both BCG recombinants. I found that the mIFN-γ is not stable in the Dubos-ADC medium under the BCG growth conditions.

I chose murine leishmaniasis as a model to test whether the cytokines expressed by various BCG recombinants would modulate the effects of BCG. Initial experiments testing the effects of vaccination with huIL-2-expressing-rBCG (BCG-pMV306-CII), huTNF-α-expressing rBCG (BCG-pMV261-CIII) and mIFN-γ-expressing rBCG (BCG-pMV261-CI) in the *L. major* infection in BALB/c mice demonstrated that: the development of cutaneous lesions in BALB/c mice immunized with BCG recombinants expressing TNF-α or mIFN-γ was significantly reduced compared to the controls receiving the normal BCG, while the lesion size in the mice immunized with IL-2-expressing BCG was similar to that of control mice. In addition, The footpads of mice immunized with the mIFN-γ-expressing rBCG contain significantly fewer parasites on day 30 after infection compared to the mice given normal BCG (The

results with the use of huTNF-α and IL-2 in the leishmania studies were presented in the 95th general meeting of ASM, Washington D.C. and not shown in this thesis). Based on the initial experiment, mIFN-γ-expressing rBCG was chosen for further studies (Chapter IV). Further studies demonstrated that vaccination with the mIFN-γ-secreting rBCG could either protect or exacerbate the *L. major* infection, depending on the timing of the vaccination and infection.

In addition, I performed *in vitro* mutagenesis on two glycosylation sites of mouse interleukin-5 (mIL-5) to make it more homogenous for analysis of mIL-5 spatial structure by X-ray diffraction. While the mutated mIL-5, expressed in the Baculovirus system, showed more homogeneity than the wild type mIL-5, it retained the same biological activity. From one mutated mIL-5, two crystal forms were obtained, diffracting to beyond 2.5 A. resolution. The study of mIL-5 spatial structure is in progress.

2. Implications from my studies

Even though BCG is the most widely used vaccine for the prevention of tuberculosis worldwide and more than 2 million people are vaccinated with BCG annually, the efficacy is highly variable from 0 to 80% (see chapter I). Control of tuberculosis and other mycobacterial infections requires a new vaccine. Various cytokines, including IL-2, IFN-γ and TNF-α, have been shown to be effective immunological adjuvants for a variety of viral, bacterial and parasitic vaccines (For a review see Heath and Playfair, 1992). Our

cytokine-expressing rBCG may be a more effective vaccine for the prevention of tuberculosis and other mycobacterial infections, since the cytokines expressed by the recombinant BCG could serve as adjuvants for mycobacterial antigens. As presented in Chapter II, the huIL-2-expressing rBCG shows limited growth in BALB/c mice, which raises a possibility of loss of immunogenicity if rBCG is cleared too early. This is a complex situation in which huIL-2 expressed by rBCG could have dual effects on the immunogenicity; on one hand, huIL-2 can enhance the immune responses to BCG antigens, but on the other hand, huIL-2 could also reduce the amount of BCG present. The immunogenicity should depend on the balance between the two opposing effects. Our preliminary results showed that antibodies against mycobacterial antigens were increased two times in the mice immunized with the huIL-2-expressing rBCG compared with the mice immunized with BCG (data not shown). Before we draw any conclusions about whether our cytokine-expressing rBCGs may be used as effective vaccines, more experiments by comparing the immunogenicity of our cytokine-expressing rBCGs to the BCG Pasteur strain are needed.

As discussed in the Chapter I, pathogenic mycobacteria (*M. tuberculosis* and *M. leprae*) are facultative intracellular pathogens that mainly live and multiply in non-activated macrophages. Before mycobacteria can be destroyed by macrophages, these cells must be activated by T lymphocytes and/or lymphokines. IL-2, IFN-γ and TNF-α are three major cytokines, which are directly and/or indirectly involved in the activation of macrophages, and therefore

play an important role in the host defense against mycobacterial infections. It has been shown by many studies that administration of these cytokines can increase the host resistance to mycobacterial infections. Our cytokine-expressing rBCGs have potential use in the treatment of various mycobacterial and other intracellular bacterial infections.

BCG instillation is the treatment of choice for recurrence of superficial bladder cancer (Soloway and Perito, 1992). As described in chapter I, many cytokines, such as IL-2, TNF-α and IFN-γ, have been implicated in the anti-tumor effects of BCG treatment. The combination of IL-2 and BCG in the therapy of superficial bladder cancer is more effective than either IL-2 or BCG alone in both a mouse model (Ikemoto *et al.*, 1991) and in a clinical trial (Cockett *et al.*, 1991). Our cytokine-expressing BCG, especially huIL-2-expressing rBCG, should be tested for efficiency in a model of cancer.

One of the important implications of our studies is that our recombinant BCGs may be safer vaccines for the prevention of mycobacterial infection in AIDS or other immunocompromised patients. Mycobacterial infections are commonly seen in AIDS patients, and BCG vaccination has been suggested to prevent these infections (Quinn, 1989). The immunosuppression in AIDS patients raises the issue of safety of BCG vaccination. BCG local reactions and disseminated diseases caused by BCG immunization have already been described in HIV-infected children and adults (Nousbaum *et al.*, 1986; Tarantola, 1987). Our cytokine-expressing BCG may enhance

the immune response of AIDS patients, and therefore, prevent disease by BCG. As described above, expression of IL-2, IFN-γ and TNF-α in vaccinia virus can render these viruses highly attenuated; therefore, immunodeficient mice, such as nude mice and sublethally irradiated mice, are readily able to control the growth of these recombinant viruses. Although we have not tested whether our cytokine-expressing BCG recombinants are attenuated in immunodeficient mice, we have shown that the huIL-2-secreting BCG recombinants show limited growth in BALB/c mice (Kong and Kunimoto, 1995). We infer from these studies that the expression of cytokines in BCG may provide a way to attenuate BCG for vaccination against mycobacterial infection in HIV or other immunocompromised patients. Theoretically, cytokine-expressing BCG may not only render the BCG vaccine safer, but also could overcome some of the immunological defects caused by HIV infection and therefore, increase the vaccine efficacy.

3. Problems with the expression of cytokines in BCG

One of the difficulties in the expression of huIL-2, huTNF-α and mIFN-γ in BCG is to obtain the BCG recombinants harboring the extrachromosomal cytokine-expressing plasmids constructed from the vector pMV261. As presented in chapter II (Table 2.2), no colonies could be obtained from the electroporation of all three plasmids using the vector pMV261, having either the huIL-2 gene or the huIL-2 gene linked to the α-antigen signal peptide under the control of either the α-antigen promoter or the hsp60 promoter. The

plasmid pMV261-CIII (huTNF-α), in which the huTNF-α gene is fused to the hsp60 regulatory sequences, can be maintained in BCG cells. In contrast, pMV261 plasmids having huTNF-α linked to the signal peptide of the BCG α-antigen under the control of either the α-antigen promoter or the hsp60 promoter cannot be maintained in BCG cells. While the BCG cells harboring the plasmid pMV261-CI (mIFN-γ), in which the mIFN-γ gene is under the control of a weaker promoter (α-antigen promoter), is viable, the other two mIFN-γ expressing plasmids, in which the mIFN-γ gene is under the control of the hsp60 promoter, appeared to be lethal to BCG cells. On the other hand, all expression cassettes cloned in an integrative vector pMV306 can be maintained in the genome of BCG. This provides examples of being able to maintain foreign genes, which are not easily established with plasmid vectors, by using an integrative vector in BCG just as described with other bacterial systems (Saunder *et al.*, 1984).

A likely explanation for this phenomenon is over-expression lethality (Burlein *et al.*, 1994). Since the extrachromosomal plasmid pMV261 can replicate to approximately five copies per genome equivalent in BCG cells, while pMV306 only integrates one copy into the att B sites of BCG, the higher protein expression in pMV261-containing recombinants may be lethal to the host. Other researchers have found the same phenomenon. For example, Stover *et al.* (1991) found that gp120 gene of HIV-1 could be expressed from the hsp 60 promoter only when it was integrated, and gp120 could be expressed in multicopy plasmid vectors when weaker

promoters were used. As described above, mIFN-γ under the control of a weaker promoter (α-antigen) in plasmid pMV261-CI (mIFN-γ) could be maintained in BCG, while other two plasmids, in which the mIFN-γ was under the control of a strong promoter hsp60, could not be established in BCG.

On the other hand, the expression level of cytokines in various BCG recombinants seems to be the result of more than the regulatory sequences and the copy number of genes. rBCG, harboring extrachromosomal pMV261-CIII (huTNF-α), expressed 600 times more huTNF-α than rBCG, having pMV306-CIII (huTNF-α) integrated (Table 3.3 of Chapter III). The plasmid ratio in these two BCG recombinants should only be 1 to 5 and the expression cassette inserted into the two expression plasmids is the same. The reason for this difference is unknown. It is possible that some DNA sequences around the integration site of pMV306-CIII (huTNF-α) interferes with the expression of huTNF-α when the huTNF-α gene is integrated into the BCG genome.

To express cytokines in BCG, two different expression vectors were used throughout my studies; one is the integrative vector pMV306 and another is the extrachromosomal vector pMV261. The rBCG harboring pMV261 generally expresses a much higher amount of protein than rBCG having pMV306 (see above for an example), possibly due to the high copy number of pMV261 in rBCG . But on the other hand, some genes can only be expressed using the integrative vector pMV306. Another advantage of using the

integrative vector is its stability in the absence of selection when the recombinant BCG vaccines are going to be used *in vivo*.

Another potential problem with the expression of cytokines in BCG is that the cytokines secreted by BCG recombinants may not be functional *in vivo*. BCG is facultatively intracellular bacterium, and macrophages are the major habitat. Analyses of the early events in the phagocytosis of mycobacteria *in vitro* by McDonough *et al.*, (1993) have indicated that the majority of BCG cells reside in the fused phagolysosomes 2 hours after infection, and then the BCG cells remain in the vacuoles until most of them are degraded. Therefore, it is possible that once the BCG cells are engulfed by macrophages, the cytokines, secreted by BCG recombinants, would not have any function inside macrophages. The cytokines may also be degraded inside the macrophage, and the degraded peptide may be secreted out and interfere with the normal immune response. I found that our mIFN-γ-secreting BCG exacerbated the *L. major* infection in BALB/c mice at a late infection stage (Chapter IV). It is possible that at a late time of infection, degraded mIFN-γ peptide interferes with the function of macrophages, and exacerbates the *L. major* infection, although this hypothesis does not account for the early protective effect being greater than that of BCG alone.

A potential problem is the use of human cytokines in a mouse model. Both huIL-2 and huTNF-α function in mice, since the bioassay of huIL-2 is based on the growth of an IL-2 dependent murine cell line HT2 (Chapter II), while the bioassay of huTNF-α is based on the cytolytic effect of huTNF-α on a murine cell line

L-929-8 (Chapter III). It is possible that an immune response against the cytokines will occur but as can be seen, if it has occurred it has not neutralized the activity of the cytokine *in vivo*, although I would not know whether it reduced the effect of the cytokines. I expressed huIL-2 and huTNF-α in BCG instead of expressing mIL-2 and mTNF-α in order to be able to use these rBCGs in humans if warranted in the future.

4. Prospects.

As discussed in section two of this chapter, expression of cytokines in rBCG provides a new means to deliver cytokines to the host immune system. Cytokine-expressing BCG recombinants have potential in the treatment of various intracellular bacterial infections, and probably some tumors. The cytokine-expressing rBCG may be a more effective vaccine for tuberculosis, and the secreted cytokines may act as adjuvants to improve the immune response against coexpressed proteins in BCG. But before they can be used in clinical trials, many studies have to be done to optimize the levels of cytokines expressed by various BCG recombinants. At the same time, more experiments are needed to understand the mechanisms involved in the immunostimulatory effects of various cytokine-expressing BCG recombinants in an animal model.

As I presented in chapter II, huIL-2-expressing rBCG shows limited growth in BALB/c mice, but no similar experiments were done to monitor the growth rate of mIFN-γ-expressing rBCG and huTNF-α-expressing *in vivo*. The reason is that the rBCG expressing

huTNF-α and mIFN-γ grow more slowly *in vitro* than the rBCG harboring only a vector (data not shown), therefore there is no appropriate control to compare the *in vivo* growth rate of these rBCG.

The following experiments are suggested for future studies.

a). Improvement of the cytokine-expressing BCG recombinants.

Although various BCG recombinants expressing huIL-2, huTNF-α and mIFN-γ have been developed, there is room for improvement. As already demonstrated in huIL-2 expressing rBCG, the dose of cytokine produced is an important factor in determining the stimulatory effects of the rBCG on the host immune response. Since I do not know what is the optimal dose of the cytokine expressed by the BCG recombinants, to provide the suitable immune stimulatory effect, more BCG recombinants expressing different levels of cytokines should be made and then tested in animal models. To achieve this goal, the activity of promoters and other regulatory DNA elements from various mycobacterial genes should be tested using huTNF-α as a reporter gene, and then the optimal promoters could be used in driving the expression of other cytokine genes. As the hsp60 promoter is a stronger promoter than the α-antigen promoter in driving the expression of all three cytokines, promoters from other genes may show different activity in directing gene expression. Since changing AUG to GUG in the hsp60-huTNF-α fusion gene can enhance the expression of huTNF-α, changing the initiation codon would be a useful strategy to alter the expression level of other cytokines in BCG.

Another way to change the levels of gene expression is to use different expression vectors, as different vectors may replicate to different copy numbers in mycobacteria, and therefore, the gene cloned in different vectors may express different amount of proteins. Our lab has recently obtained an integrative vector pUS903 from Dellagostin *et al.* (1993). The vector pUS903 contains two copies of the insertion sequence IS900, which allows the vector and cloned gene to be transposed into the mycobacterial genome. Generally, one to five copies of this vector can be integrated into the genome. Insertion of my expression cassettes into this vector, followed by electroporation of the constructed expression plasmids into BCG would allow us to obtain a large number of BCG recombinants with different copies of genes inserted in the BCG genome. These BCG recombinants may express different levels of cytokines, allowing us to select the BCG recombinants expressing the optimal dose of cytokines.

b). Use of the cytokine-expressing BCG recombinants.

First of all, experiments should be carried out to test whether the cytokine-expressing BCG recombinants could be used as more efficient vaccines against tuberculosis in mouse or guinea pig models. It is important to use an aerosol chamber to challenge the vaccinated mice or guinea pigs through the respiratory route, which is the natural route of *M. tuberculosis* infection in humans. At the same time, the humoral and cell-mediated immune response against

mycobacterial antigens should be measured from mice vaccinated with cytokine-expressing rBCG or control BCG.

Second, we should test whether cytokines expressed by BCG recombinants could act as an adjuvant to improve the efficacy of coexpressed proteins. To do this, a BCG recombinant expressing both a cytokine gene and a foreign gene could be made by either cloning two genes into the same vector or cloning two genes into two different vectors, which carry a different selection marker. This BCG recombinant, expressing a cytokine and a foreign antigen, could be tested in a mouse model to study the humoral and cell-mediated immunostimulatory effects of expressed cytokines on the expressed foreign antigen. The humoral immune response would be judged by the production of specific antibody. The cell-mediated immune response would be measured by the T cell functions of the immunized animal, which could be assayed using the T cell proliferation assay, cytolytic activity assay and production of various cytokines from T cells after specific antigen stimulation.

Third, it has been demonstrated that expression of cytokines in vaccinia virus provides one way to attenuate this virus as a safer vaccine for immunocompromised individuals. We should test whether our cytokine-expressing BCG recombinants are attenuated in immunodeficient animals, like nude mice. As discussed in the last section, an attenuated BCG may provide a safer vaccine for vaccination in AIDS patients against mycobacterial infections.

Finally, our cytokine-expressing BCG recombinants have potential in the treatment of some tumors. The IL-2-secreting BCG

recombinants in particular have great potential in the treatment of bladder cancer as discussed in Chapter II. Experiments using our cytokine-expressing BCG in the treatment of certain tumors should be tested in some animal models.

c). Immunological studies.

As described in chapter II, administration of the huIL-2-secreting BCG into BALB/c mice can limit the growth of BCG *in vivo*. Although the data indicates that the inhibition of BCG growth in BALB/c mice is likely due to augmentation of the host immune response by secreted huIL-2, the mechanism(s) involved are unknown. To test whether the huIL-2 secreted by rBCG is responsible for the inhibition of BCG replication *in vivo*, anti-huIL-2 antibodies can be injected into the huIL-2-expressing rBCG-vaccinated mice to neutralize the effect of huIL-2. Since T cells play a critical role in the host defense against mycobacterial infection, the function of T cells should be studied. To test whether the function of T cells was enhanced by the huIL-2-expressing rBCG, T cells can be isolated from the spleens or lymph nodes of mice inoculated with huIL-2-secreting rBCG or BCG controls. Assays for T cell proliferation or secretion of various cytokines after stimulation with mycobacterial antigen could be carried out.

When studying the effects of our mIFN-γ-secreting BCG recombinant on the *L. major* infection in BALB/c mice, we found that vaccination with the mIFN-γ-secreting rBCG could either protect or exacerbate the *L. major* infection, depending on the timing of the

vaccination and infection. The mechanism(s) involved in the exacerbation of *L. major* infection by our mIFN-γ-secreting BCG is unknown. Since *L. major* only replicates in the macrophage, the function of macrophages is essential for the control of *L. major* infection. As discussed in the Chapter IV, exacerbation of *L. major* infection may be due to the impairment of macrophage function by the mIFN-γ-secreting rBCG at a late infection stage. To test the function of macrophages in the infected mice, macrophages can be isolated from the spleens or peritoneum of immunized mice. The ability of macrophages to kill tumor cells or *L. major* can be tested *in vitro*. Their ability to produce NO after stimulation with LPS and mIFN-γ can be also determined, since production of NO is the major mechanism by which macrophages kill intracellular pathogens.

In summary, expression of cytokines in BCG provides a novel means to deliver cytokines to the host immune system. The various cytokine-expressing BCG recombinants I constructed during my Ph.D. studies have great potential to be used as more efficient vaccines against infection by *M. tuberculosis* or *M. leprae*, or could be used in the treatment of mycobacterial infections. In addition, delivery of cytokines to the microenvironment of the BCG infection provides a powerful tool to study the effects of various cytokines in mycobacterial infections.

BIBLIOGRAPHY

Balkwill, F.R., and F. Burke. 1989. The cytokine network. Immunology Today 10: 299-304.

Bermudez, L.E., and G. Kaplan. 1995. Recombinant cytokines for controlling mycobacterial infections. Trends Microbiol. 23: 22-27.

Bogdan, C., A. Gessner, and M. Rollinghoff. 1993. Cytokine in leishmaniasis: A complex network of stimulatory and inhibitory interactions. Immunobiol. 189: 356-396.

Bottger, E.C. 1994. Resistance to drugs targeting protein synthesis in mycobacteria. Trends microbiol. 2: 416-421.

Cole, S.T. 1994. *Mycobacterium tuberculosis*: drug-resistance mechanisms. Trends Microbiol. 2: 411-415.

Denich, K., P. Borlin, P.D. O'Hanley, M. Howard, and A.W. Heath. 1993. Expression of the murine interleukin-4 gene in an attenuated aroA strain of *Salmonella typhimurium*: persistence and immune response in BALB/c mice and susceptibility to macrophage killing. Infect. Immun. 61: 4818-4827.

Flesch, I.E.A., and S.H.E. Kaufmann. 1993. Role of cytokines in tuberculosis. Immunobiol. 189: 316-339.

Heath, A.W., and J.H.L. Playfair. 1992. Cytokines as immunological adjuvants. Vaccine 10: 427-434.

Hinuma, S., M. Hazama, A. Mayumi, and Y. Fujisawa. 1993. A novel strategy for converting recombinant viral protein into high immunogenic antigen. FEBS Letters 288: 138-142.

Howard, M.C., A. Miyajima, and R. Coffman. 1993. T cell-derived cytokines and their receptors. In: Fundamental Immunology, 3rd edition, edited by W.E. Paul. Raven Press. Ltd., New York. pp763-800.

Karupiah, G., R.V. Blanden, and I.A. Ramshaw. 1990. Interferon-γ is involved in recovery of athymic nude mice from recombinant vaccinia virus/interleukin 2 infection. J. Exp. Med. 172: 1495-1503.

Locksley, R.M., and P. Scott. 1991. Helper T cell subsets in mouse leishmaniasis induction, expression and effector function. Immunol. Today 12: A58-A61.

Modlin, R.L. 1994. Th1-Th2 paradigm: Insights from leprosy. J. Invest. Dermotol. 102: 828-832.

Mosmann, T.R., and K.W. Moore. 1991. The role of IL-10 in cross regulation of TH1 and TH2 responses. Immunology Today 12: A49-A53.

Mosmann, T.R., and R.L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7: 145-173.

Nousbaum, J.B., M. Garre, J.M. Roles, B. Garo, and J.J. Larzul. 1986. Deux manifestations inhabituelles d'une infection par le virus

LAV-HTLV-III BCGite et varicelle palmonaire. Rev. Pneumol. Clin. 42: 310-1.

Orme, I.M., A.D. Roberts, J.P. Griffin, and J.S. Abrams. 1993. Cytokines secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J. Immunol. 151: 518-525.

Quinn, T. C. 1989. Interactions of the human immunodeficiency virus and tuberculosis and the implications for BCG vaccination. Rev. Infect. Dis 2: S379-S384.

Ramshaw, I.A., J. Ruby, and A. Ramsay. 1992. Cytokine expression by recombinant viruses a new vaccine strategy. Trends Biotechnol. 10: 424-426.

Ramshaw, I.A., M.E. Andrew, S.M. Phillips, D.B. Boyle, and B.E.H. Coupar. 1987. Recovery of immunodeficient mice from a vaccinia virus/IL-2 recombinant infection. Nature 329: 545-546.

Rosenberg, S.A., B.S. Packard, P.M. Aebersold, D. Solomon, S.L. Topalian, S.T. Toy, P. Samon, M.T. Lotze, J. C. Yang, C.A. Seipp, C. Simpson, C. Carter, S. Bock, D. Schwartzentruber, J.P. Wei, and D.E. White. 1988. Use of tumor-infiltrating lymphocytes and interleukin 2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N. Engl. J. Med. 319: 1676-1680.

Rosenberg, S.A., M.T. Lotze, L.M. Muul, S. Leitman, A.E. Chang, S.E. Ettinghausen, Y.L. Matory, J.M. Skibber, E. Shiloni, J.T. Vetto, C.A. Seip, C. Simpson, and C.M. Reicher. 1985. Observation on the systemic administration of autologous lymphokine-activated killer

cells and recombinant interleukin 2 to patients with metatastic cancer. N. Engl. J. Med. 313: 1488-1492.

Sambhi, S.K., M.R.J. Kohonen-Corish, and I.A. Ramshaw. 1991. Local production of tumor necrosis factor encoded by recombinant vaccinia virus is effective in controlling viral replication *in vivo*. Proc. Natl. Acad. Sci. USA. 88: 4025-4029.

Stover, C.K., V.F. De la Cruz, T.R. Fuerst, J.E. Burlein, L.A. Benson, L.T. Bennett, G.P. Bansal, J.F. Young, M.H. Lee, G.F. Hatfull, S.B. Snapper, R.G. Barletta, W.R. Jacobs, Jr., and B.R. Bloom. 1991. New use of BCG for recombinant vaccines. Nature 351: 456-460.

Tarantola, D., and J.M. Mann. 1987. Acquired immunodeficiency syndrome (AIDS) and expanded programs on immunization. Special program on AIDS. Geneva: World Health Organization.